Synergistic Activities of the Selected Antibiotics of Fluoroquinolones, β-Lactams, Aminoglycosides, Glycopeptides and Streptogramins Against Gentamicin-Resistant *Enterococcus faecalis* and *Enterococcus faecium*

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Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

2005

Abstract

There is a need for new agents or combination of agents for treatment of serious infections caused by gentamicin and vancomycin-resistant enterococci, which may be resistant to all available antimicrobial agents. The enterococci resistance against antimicrobial agents may be due to both intrinsic and acquisition of the resistance genes to counter antibiotics which were once effective. However, there have been only a few antimicrobial agents which have any activity against these resistant organisms and it is likely that the pathogens will soon become resistant to these as well. One strategy to preserve the efficacy of these new compounds may be to use them in combination. Similarly, combination therapy might be the only strategy that might be effective against some strains, particularly those responsible for endocarditis, intra-abdominal sepsis and urinary tract infections. It is therefore, important to study further the combination of agents against enterococci especially those with high-level resistant to gentamicin to establish the synergistic activities of the antibiotics. Also to identify by PCR technique the existence of some resistance genes involved, for example, in gentamicin and ciprofloxacin antibiotics. The aim of this study was to investigate synergistic activities of the combined antibiotics against the gentamicin resistant *Enterococcus faecalis* and *Enterococcus faecium* and to establish the existence of some genes involved in the resistance of enterococci against gentamicin and ciprofloxacin. The total of 81 clinical isolates were collected for the study and all were found to be resistant to gentamicin (MICs range 32->256mg/l). 50 isolates were found to be resistant to ciprofloxacin with MICs range 64->256mg/l. The synergistic activities of the antibiotics combinations were established against

E.faecalis and *E.faecium* clinical isolates. The combination between amoxicillin and gentamicin against *E.faecalis* resulted into synergy with MIC at 90% of 0.5mg/l and for *E.faecium* MIC at 90% was 2mg/l. Vancomycin and gentamicin combination had MIC at 90% of 2mg/l for *E.faecalis* and 1mg/l for *E.faecium*. Teicoplanin and synercid combination showed synergistic activity of MIC at 90% of 0.5mg/l for *E.faecalis* while *E.faecium* had also 0.5mg/l. The combination between teicoplanin and ciprofloxacin had synergy with MIC at 90% of <0.25mg/l for *E.faecalis* and *E.faecium* had the same activity MIC at 90% of <0.25mg/l. The combination between amoxicillin and synercid resulted into synergistic activity at MIC at 90% of 4mg/l for *E.faecalis* and also 4mg/l for *E.faecium*. The antagonistic activity was observed between the combination of vancomycin and synercid with MIC at 90% of 32mg/l for *E.faecalis* and MIC at 90% of 16mg/l for *E.faecium*. The combination between synercid and ciprofloxacin resulted into synergistic activity at 90% MIC of 4mg/l for *E.faecium*. The checkerboard test of the combination between synercid and ciprofloxacin against four clinical isolates of *E.faecium* resulted into FIC indices of 0.4 for isolate D002 and 0.4 for isolate G051. While isolate 788/5/95 had 0.3 and NCTC12202 had also 0.3. These indices confirm the synergistic activity of the combined antibiotic of the two drugs. Of 27 clinical isoaltes tested for the presence of aminoglycoside modifying enzymes using PCR technique, only 8 (2 *E.faecium* and 6 *E.faecalis*) showed the positive results of the presence of AME. However, the presence of the AME did not prevent the synergistic activities of the combined drugs against *E.faecalis* and *E.faecium*. The PFGE study showed the heterogeneous existence of these gentamicin resistance isolates from RIE. Also, of the isolates (all *E.faecalis*) tested for the presence of *gyr*A and *par*C among the isolates with MICs >256mg/l and one with MIC of 0.5mg/l against ciprofloxacin, 6 (5 with MICs >

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* = For different Isolates

Declaration

The experiments and composition of this Thesis are the work of the author unless otherwise stated.

Acknowledgments

Firstly, I would like to thank Professor Sebastian GB Amyes for his helpful direction and guidance throughout my study. Also his understanding with regards to my part-time job for my survival during the course of my studies in this University. I would like also to thank Dr. Elumogo Ngozi for her useful discussions and support. Thanks to Dr.Devula Kenna and Vicki for their support and helpful advice. I am also grateful to my Kids for their patience throughout my study in Edinburgh. This thesis is dedicated in the memory of my parents (Okello Arum and Anying) who passed away more than twenty years ago and my Nephew (Otto-Okello L) who was killed during the regime of Iddi Amin in Uganda. May almighty God keep their hearts in heaven. Thanks to all my relatives who gave me a lot of helps during my hard time periods. I am extremely grateful to my niece (Margret Amono) for her kind support and making sure always that I am OK during my study in UK. I am also grateful to my colleagues within the Division of Medical Microbiology and especially those within the Molecular Chemotherapy research group for their support.. A very kind support from Robert Brown is also acknowledged.

Publications and Presentations

Obwona AB, Amyes SGB and Elumogo TN, **2000**. The Synergism between synercid and ciprofloxacin against gentamicin and vancomycin resistant enterococci, abstract 107. In: Program and abstracts of the 1st International ASM Conference on Enterococci: pathogenesis, Biology and Antibiotic Resistance. American Society for Microbiology, Washington, DC.

Abbreviations

Ala	-	Alanine or alanyl
AAC	-	Aminoglycoside acetyltransferase
AAD	-	Aminoglycoside adenyltransferase
AME(s)	-	Aminoglycoside modifying enzyme(s)
Amox	-	Amoxicillin
ANT	-	Adenyltransferase
APH	-	Aminoglycoside phosphotransferase
AP-PCR	-	Arbitrary primed-PCR
Asp	-	Aspartic acid
AS	-	Aggregation substance
ATCC	-	American type culture collection
ATP	-	adenosine 5'-triphosphate
Aug	-	augmentin
BA	-	blood agar
BHI(A)	-	Brain heart infusion (agar)
bp	-	Base pair
CFU	-	Colony forming unit
Cip	-	ciprofloxacin
DNA	-	deoxyribonucleic acid
dATP	-	2'-deoxyadenosine 5'-triphosphate
dCTP	-	2'-deoxycytidine 5'triphosphate
dGTP	-	2'-deoxyguanosine 5'-triphosphate

dTTP - 2'-deoxythymidine 5'-triphosphate

EDP1 - Energy dependant plasma 1

EDP11 - Energy dependant plasma 11

EIP - Energy independent plasma

EDRN - *Enterococcus durans*

EDTA - ethylenediaminetetraacetic acid

*Efa*A - *Enterococcus faecalis* antigen A

EFCL - *Enterococcus faecalis*

EFCM - *Enterococcus faecium*

FIC - Fractional inhibitory concentration

Gen - gentamicin

Glu - glutamate

Gm^r - getamicin resistant

GRE - Glycopeptide resistant enterococci

HLGRE - High-level gentamicin resistant enterococci

HLGR - High-level gentamicin resistance

MLGR - Moderate-level gentamicin resistance

HPLC - High performance liquid chromatography

ile - isoleucine

IS - insertion sequence

ISTA - Isosensitest agar

Lin - linezolid

L-PCR - long-template PCR

log - logarithm

MIC(s) - minimum inhibitory concentration (s)

MHA - Mueller-Hinton agar

Mox - moxifloxacin

NCTC - National collection of type cultures

NNIS - National Nosocomial Infection Surveilance

PBP - Penicillin binding protein

PCR - polymerase chain reaction

PFGE - Pulsed-field gel electrophoresis

Pip - piperacillin

Pro - proline

psi - pounds per square inch

Q/D - quinopristin/ dalfopristin

QRDR - quinolone resistant determining region

RAPD - Random amplified polymorphism DNA

RIE - Royal Infirmary of Edinburgh

RNA - ribonucleic acid

rRNA - ribosomal ribonucleic acid

Ser - serine

Syn - synercid

Taz - tazocin

Teic - teicoplanin

Tn - transposon

Val - valine

Van - vancomycin

U - units of enzyme

UV - ultraviolet

CHAPTER 1

1. INTRODUCTION

1.1 *Enterococcus*

The prevalence of enterococci as nosocomial pathogens has increased over the past 15 years. They are now well established as the third most common etiology of endocarditis (Murray and Weinstock, 1999) and are second most common cause of surgical wounds infections and nosocomial urinary tract infections and the third most common cause of nosocomial bacteraemias in USA (Tenover *et al*, 1993; Emori and Gyanes, 1993). However, enterococcal resistance to antimicrobial agents has emerged resulting in serious therapeutic difficulties.

1.1.1 Historical View

" *Enterococcus*" was first termed as "*Micrococcus zymogenes*" by MacCallum and Hastings in 1899 at Johns Hopkins Hospital-USA, identified in the fatal case of endocarditis. In 1906, the name " *Streptococcus faecalis*" was coined by Andrews and Horder referring to a faeces organism. In 1930s, enterococci were grouped into group D streptococci based on serological typing by Rebecca Lancefield showing the difference in the cell-wall polysaccharide antigen (Lancefield, 1933). Sherman created two groups of streptococci in 1937 and named one group that grew at 10°C and 45°C, in broth containing 6.5% NaCl, at pH9.6 and survived heating at 60°C for 30 minutes as

Streptococcus faecalis and *Streptococcus faecium*. The second group that did not grow in broth containing 6.5% NaCl was named as *Streptococcus bovis* and *Streptococcus equinus*. In 1970, Kalina proposed the generic name "*Enterococcus*", which included two species (ie *E.faecalis* and *E.faecium*). Schleifer and Kilpper-Balz gave genetic evidence in 1984 that *Streptococcus faecalis* and *Streptococcus faecium* were different from other members of the *Streptococcus* based on DNA-DNA and DNA-RNA hybridization studies and therefore they should be in a separate genus (ie *Enterococcus*). The genus *Enterococcus* emphasises the intestinal origin of gram-positive diplococucus (Murray, 1990).

1.1.2 *Enterococcus* species

Since Kalina proposed in 1970 that genus "*Enterococcus*" be named based on cellular and phenotypic characteristics also confirmed by Schleifer and Kilpper-Balz in 1984 based on DNA-DNA, DNA-RNA hybridization and 16s rRNA sequencing , 17 other species of the same genus have been included based on chemotaxonomic and phylogenetic studies (Devriese *et al*, 1993). However, many of the recently included species (Table 1.1) based on 16s rRNA sequencing studies do not give typical reactions of the genus described by Sherman (Sherman, 1937). The descriptions of *E. seriolicida* and *E.solitarius* and the phylogenic studies indicate that they are more closely related to *Lactococcus* and *Tetragenococcus* species respectively (Collins et al, 1990; Kusuda, 1991).

Table 1.1 List of *Enterococcus* species described.

Present name	Description of the species	16S rRNA gene sequencing
***Enterococcus* species**	***By**	**By**
faecalis	Schleifer & Kilpper-Balz (1984)	Williams *et al*, 1991
faecium	Schleifer & Kilpper-Balz (1984)	Williams *et al*, 1991
avium	Collins *et al*, (1984)	Williams *et al*, 1991
casseliflavus	Collins *et al*, (1984)	Williams *et al*, 1991
gallinarum	Collins *et al*, (1984)	Williams *et al*, 1991
durans	Collins *et al*, (1984)	Williams *et al*, 1991
malodoratus	Collins *et al*, (1984)	Williams *et al*, 1991
hirae	Farrow & Collins (1985)	Farrow & Collins, 1985
mundtii	Collins *et al*, (1986)	Collins *et al*, 1986
pseudoavium	Collins *et al*, (1989)	Collins *et al*, 1989
raffinosus	Collins *et al*, (1989)	Collins *et al*, 1989
solitarius	Collins *et al*, (1989)	Patel *et al*, 1998
cecorum	Williams *et al*, (1989)	Devriese *et al*, 1983
columbae	Devriese *et al*, (1990)	Devriese *et al*, 1990
saccharolyticus	Rodrigues & Collins (1990)	Rodrigues & Collins, 1990
sulfureus	Martinez-Murcia & Collins (1991)	Martinez-Murcia & Collins (1991)
seriolicida	Kusuda *et al*, (1991)	Domenech *et al*, 1993
dipar	Collins *et al*, (1991)	Collins *et al*, 1991
flavescens	Pompei *et al*, 1992	Patel *et al*, 1998

*Adapted from Devriese *et al*, 1993

1.1.3 Habitat of Enterococci

The nature of these bacteria, which allows them to grow and survive in harsh environments, makes them persist almost everywhere. They can be found in soil, food, water, plants, birds, insects, humans and animals (Devriese *et al*, 1992; Dutka and Kwan, 1978; Kibbey *et al*, 1978). In humans as well as in other animals, the enterococci inhabit the gastrointestinal tracts and female genital tracts. *Enterococcus faecalis* is one of the most common bacteria isolated from gastrointestinal tracts of humans. *E.faecium* is commonly found in the gastrointestinal tract of humans as well (Endtz *et al*, 1997).

1.1.4 Identification of the *Enterococcus* species

The identification of enterococci to species level plays one of the crucial roles in proper patient management and for epidemiologic purposes. Although the current tests can identify most of the *Enterococcus* species related to human infections, many of the recently established enterococcal species could not be readily identified by such tests. The two species (ie *E.faecalis* and *E.faecium*) have common characteristics that allow them to be distinguished from other catalase-negative, gram-positive facultative cocci. They include ability to grow at 10°C and 45°C, at pH 9.6, and 6.5%sodium chloride broth (Schleifer and Kilpper-Balz, 1984) and also the presence of Lancefield group D antigen. However, newly established enterococcal species fail to react in the presence Lancefield group D antisera and also fail to grow in the conditions that show characteristic of *E.faecalis* and *E.faecium* (Collins *et al*, 1989; Devriese *et al,* 1990; Facklam and Collins, 1989; Martinez-Murcia and Collins,1991). However, not only enterococci show positive reactions to

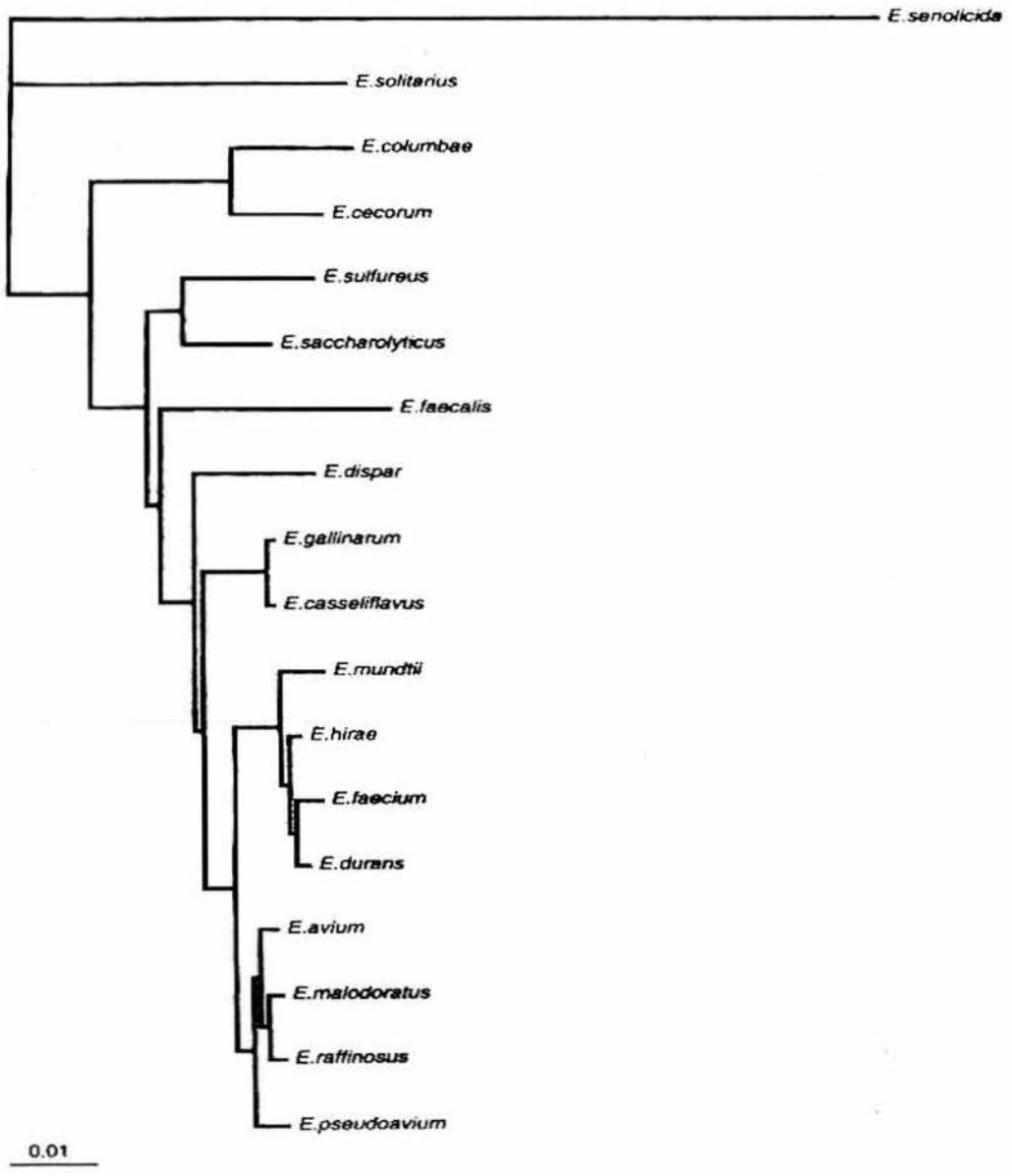
these tests, several streptococcal species, pediococci, lactococci, aerococci and leuconostoc can also react positively with the tests (Facklam *et al*, 1989). Other routine tests include hydrolysis of esculin in the presence of 40% bile, presence of pyrrolidonyl arylamidase (PYR), tests for urase, β-glucosidase and β-glucuronidase activities and an array of carbohydrate acidification tests (Devriese *et al*, 1993). These tests remain valid when seeking only the classical *Enterococcus* species.

1.1.4.1 Intragenic Regions of 16s rRNA gene sequences-useful for Identification of enterococcal species

The tests for identification of enterococcal species mentioned in section 1.1.4 remain valid when seeking only classical *Enterococcus* species. The molecular technique such as PCR devised by Dutka-Malen *et al*, 1995 fails to identify some enterococcal species outside the reach of the primers and it is necessary, therefore, to include molecular technique such as 16s rRNA gene sequences for enterococcal species identification. All the known enterococcal species have had their small-subunit 16s rRNA gene sequenced (Table 1.1). The analysis of the regions of 16s rRNA gene sequences shows the phylogenic relationship among the different enterococcal species based on on homology determinations (Fig 1.1). The homology values derived from 16s rRNA sequences can be used to show the percentage homology among the enterococcal species (Table 1.2) as well as useful for identification of *Enterococcus* species (Baele *et al*, 2000; Patel *et al*, 1998). The comparative analysis of sequence of *Enterococcus* species based on 16s rRNA analysis has grouped the species into three groups within the genus (Williams *et al*,1991). The first group consists of *E.durans*, *E.faecium*, *E.hivae* and *E.mundtii*. The second group consists of *E.pseudoavium*, *E.raffinosus*, *E.avium* and *E.malodoratus*. The

third group consists of *E.columbae*, *E.faecalis* and *E.saccharolyticus*, which formed distinct lines within the genus. Patel *et al*, 1998, using this technique, confirmed the identity of two nonmotile *E.gallinarium* isolates which were misidentified as *E.faecium*. Williams *et al,* 1991 showed that relatively low RNA sequence homologies between *E.faecalis* and other enterococcal species was due to phenotypic distinctiveness of type species. The identification of *E.seriolicida* as belonging to a separate genus and subsequently renaming it as *Lactococcus garvieae* (Patel *et al*,1998) indicates the usefulness of the technique. However, the precise phylogene placement of *E.solitarius* remains unclear although it appears to be related to *Tetragenococcus* (Collins *et al*, 1991). It is acceptable generally that if the 16s rRNA gene sequence of a strain is less than 97% similar to its neighbour, it indicates that it is not the same species. However, 97% or higher may indicate that it belonges to the same species.

Fig 1.1 Phylogenic Relationships of *Enterococcus* species derived from 16s rRNA sequences



Adapted from Patel R *et al*,1998.

Table 1.2 Percentage Homology Values derived from 16s rRNA sequences of enterococci species

Species	*E. solitarius*	*E. cecorum*	*E. malodoratus*	*E. columbae*	*E. dispar*	*E. gallinarum*	*E. hirae*	*E. mundtii*	*E. avium*	*E. casseliflavus*	*E. pseudoavium*	*E. sulfureus*	*E. faecalis*	*E. raffinosus*	*E. saccharolyticus*	*E. durans*	*E. faecium*
E. seriolicida	86.1	88.2	88.6	88.2	88.5	88.2	88.5	88.5	88.6	88.2	88.4	88.4	87.9	88.6	88.2	88.4	88.6
E. solitarius		93.0	93.7	93.0	93.6	94.5	93.7	93.6	93.8	94.7	93.8	93.8	94.1	93.8	94.8	93.7	93.8
E. cecorum			95.9	97.9	95.8	96.6	95.8	95.7	95.9	96.6	95.8	96.8	95.4	95.9	96.6	95.8	95.7
E. malodoratus				96.0	97.8	98.7	99.4	99.3	99.5	98.8	99.4	97.3	96.7	99.8	97.8	99.3	99.1
E. columbae					96.3	96.5	95.8	95.8	96.0	96.4	95.7	95.5	95.2	96.0	96.7	95.9	95.8
E. dispar						97.9	97.7	97.6	97.6	97.9	97.7	96.6	96.9	98.0	97.7	97.7	97.7
E. gallinarum							98.5	98.4	98.4	99.9	98.4	97.6	97.3	98.6	98.8	98.5	98.4
E. hirae								99.5	99.5	98.5	99.3	97.1	97.0	99.5	97.7	99.8	99.7
E. mundtii									99.5	98.4	99.1	97.1	96.9	99.4	97.6	99.5	99.4
E. avium										98.5	99.5	97.3	96.6	99.7	97.7	99.4	99.2
E. casseliflavus											98.6	97.7	97.3	98.8	99.0	98.4	98.3
E. pseudoavium												97.3	97.0	99.5	97.9	99.2	99.1
E. sulfureus													96.4	97.3	97.9	97.0	96.9
E. faecalis														96.8	97.4	97.0	97.3
E. raffinosus															97.9	99.4	99.2
E. saccharolyticus																97.7	97.5
E. durans																	99.8

Adapted from Patel R *et al*, 1998

1.2 Enterococcal Infections

Most of the clinical infections are due to either *E.faecalis* or *E.faecium* (Mundy *et al*, 2000). *E.faecalis* has been found to cause around 80-90% of the enterococcal infections and *E.faecium* accounts for most of the remainder (Moellering, 1992 and Gray *et al*, 1991). The distribution of *E.faecalis* in healthy adult faeces and the presence of only 25% of *E.faecium* in individual faeces may account for the common infection by *E.faecalis* (Shah *et al*, 1987). *E.faecalis* has also been found to have virulence factors; for example, haemolysin which can lyse both bacterial and eukaryotic cell membranes (Brock and David, 1963). These factors appear not to be present in *E.faecium*. Other enterococcal species appear to be infrequent causes of infections in humans (Facklam and Collins, 1989). The use of antibiotics

such as cephalosporins, latamoxef and aztreonam which suppress much of the normal human flora and to which enterococci are resistant against them could promote enterococci to overgrow other intestinal bacteria thus enabling them to cause infections in the nosocomial settings (Morrison and Wenzel, 1986; Moellering, 1982; Chandraseken *et al,*1984). Infection may also occur in indwelling devices such as intra-vasculas carvulae; ambulatory peritioneal diaylsis catheter and biliary T-tubes in liver transplant. The most commonly found in enterococcal infections are endocarditis, urinary tract infections, bacteraemia and intra-abdominal infections. Other infections such as neonatal and meningitis in older adults caused by enterococci are rare (Eigler *et al,* 1961).

1.2.1 Infective Endocarditis

Enterococci are estimated to cause between 5 and 20% of infective endocarditis (Watanakunakorn and Burket, 1993; Megran, 1992). The disease is more common in the elderly, with an average age over 60, and in patients with degenerative valvular disease of genitourinary condition (Mandell *et al,* 1970). It may occur occasionally in children and rarely in infants (Teixeira *et al,* 1982). Enterococci can also cause endocarditis in drug addicts (Reiner *et al,* 1976). *E.faecalis* causes 85% of the cases of infection while *E.faecium* involves only in 10% of the cases.

1.2.2 Bacteraemia

The role of the enterococcal bacteraemia in morbidity and mortality has been difficult to determine because both bacteraemia and colonization are associated with mixed infections except in the cases of endocarditis or meningitis (Gullberg *et al,* 1989). Enterococci are the third most important cause of nosocomial bacteraemia in theUSA

(Schaberg *et al*, 1991). *E.faecalis* and *E.faecium* account for most infections which usually originate in the abdominal or the urinary tract. However, some cases of bacteraemia originate from the medical devices such as percutaneously-inserted central venous catheter or arterial catheter left in place for a prolonged period of hospitalization (Maki,1988). Thirty–one percent mortality rate was found to be directly attributed to enterococcal blood stream infections (Landry *et al,* 1989).

1.2.3 Urinary Tract Infections

Enterococci are involved in 10% of all urinary tract infections (Felmingham *et al*, 1992 **).** The UTI usually occurs in patients who have either gone through manipulation or underlying urogenic structural abnormalities involving the bladder , kidney and prostate (Moellering, 1992). Among the young women who are healthy, enterococci cause 5% of urinary tract infections.

1.2.4 Intra-abdominal Wound Infections

Intra-abdominal infections extend beyond the site of original including peritonitis, abscess formation and perforation diverticulitis abscess (Solomkin *et al*, 1990 **).** The infections are polymicrobial and the role of enterococci in the infections remains controversial (Nicholas and Muzik, 1992). Despite the difficulty in establishing pure enterococcal infections, it is clear that enterococci can contribute in abdominal abscess and sepsis.

1.2.5 Other Infections

Although group B streptococci and *E.coli* are the most common cause of neonatal infections, an outbreak of enterococcal sepsis in neonates has been reported in USA (

Luginbuhl *et al,* 1987). Also neonatal enterococcal sepsis was reported between 1970 and 1976 (Buchino *et al*, 1979). In addition to causing sepsis in neonates, enterococci can also cause meningitis in older children and adults due to an underlying disorder (Bayer *et al,* 1976).

1.3 Enterococcal Pathogenicity

Although enterococci cause a wide variety of diseases in humans such as urinary tract infections, infective endocarditis, nosocomial bacteraemia and biliary tract infections, the factors that determine the pathogenicity of enterococci are not well understood (Jett *et al*, 1994). The role of enterococcal virulence has been documented and several properties of enterococci have been suggested as potential virulence factors including cell surface adhesion molecules as well as extracellular products (Jett *et al*, 1994).

1.3.1 Cell Surface Adhesins

The first step in infection of human by bacteria involves adherence through surface adhesins to epithelial cells, endothelial cells or extracellular matrix. Since enterococci are normal flora of gastrointestinal tract of human, it appears as if they have adhesins that promote binding to human receptor cells of mucosal surfaces which play a role in the maintenance of colonization. In *E.faecalis*, Antigen A (*Efa*A) found in individuals with infective endocarditis plays a role as adhesions in the infection (Lowe *et al*, 1995). *E.faecalis* also has plasmid-mediated aggregation substance (pADI), which is a pheromone-inducible surface protein that promotes mating aggregate formation during

conjugation (Clewell, 1993). But, *in vitro,* the substance mediates adhesin to a variety of eukaryotic cell surfaces; for example pig's renal tubular cells (Kreft *et al*, 1992). It has been found that the substance also promotes direct opsonin-independent binding of *E.faecalis* to human neutrophils via complement receptor type3 and other receptors on the neutrophil surface (Vanek *et al*, 1999). Olmested *et al* in 1994, suggested that the aggregation substance may also enhance the ability of *E.faecalis* to associate with intestinal epithelial cells. While Huycke *et al* found in 1991 that aminoglycoside-resistant lineage of *E.faecalis* expressing cytolysin and aggregation substance proved particularly virulent.

1.3.2 Enterococcal Extracellular Products

They are substances produced or secreted by enterococci into extracellular environment such as gelatinase, hyaluronidase, cytolysin, superoxide and lipoteichoic acid.

1.3.2.1 Gelatinase

The metalloproteinase (gelatinase) from *E.faecalis* was first described more than 30 years ago (Bleiweis and Zimmerman,1964). In 1989, Mäkinen *et al* published a description of substrate specifying protease produced by *E.faecalis* OG1-10 called Metalloendopeptidase 11 obtained from a human mouth. This enzyme was capable of hydrolyzing gelatine, collagen, casein, haemoglobin and other small biological active peptides. Su *et al*, 1991, reported the sequence of protease gene,*gel*E, which encoded prezymogen now called gelatinaseE. A potential contribution of enterococcal protease to virulence was first suggested in 1975 by Gold *et al* who found that a

gelatin-liquefying of human oral from *E.faecalis* induced caries formation in germ free rats while non proteolytic strains did not. Although epidemiologic studies only suggest associations between protease production and infection, the sequence analysis of *gel*E showed amino acid similarity to zinc-metalloprotease (elastase)of *P.aeruginosa* (Fukushima *et al*, 1989; Su *et al*,1991). This enzyme (zinc-metalloprotease) is considered a virulent factor in severe pseudomonal infections, especially patients with cystic fibrosis (Doring *et al*, 1985; Vasil, 1986). The studies by Holder & Haidaris in 1979, using burned mouse model, showed that extracellular protease and elastase (zinc-metalloprotease) were the virulence factors in *P.aeruginosa* infections. G elatinase-producing strains of *E.faecalis* have been shown to contribute to virulence of endocarditis in animal model (Gutschik *et al,* 1979)

1.3.2.2 Hyaluronidase

Although the studies by Rosan and Williams in 1964, found that some enterococci (oral) especially *E.faecalis* produced hyaluronidase, less is known about the contribution of the enzyme in enterococcal virulence.

1.3.2.3 Cytolysin

It is a haemolytic, post-translationally-modified protein toxin that occurs in up to 60% of *E.faecalis* retrieved from outbreak investigations (Jett *et al*, 1992). Cytolysin is distantly related to streptolysin S and also to members of a class of bacteriocins known as lantibiotics displaying both haemolytic and bactericidal activity (Hancock & Gilmore, 2000). It is either coded within large, pheromone responsive plasmids (pADI) (Clewell, 1981) or on the chromosome (Ike and Clewell, 1992). Jett & Gilmore, 1990 found that cytolysin possesses plasmid (pADI) encoded bacteriocin which is lethal to wide range of

gram-positive bacteria. Independent studies, using different models, have found that cytolysin plays a role in *E.faecalis* infection (Callegan *et al*, 1999).

1.3.2.4 Extracellular Superoxide

It is associated with enterococcal virulence in bacteraemia (Huycke *et al,*1996 **).** Both *E.faecalis* and *E.faecium* produce extracellular superoxide during the infection by the invasive strains than the commensal isolates**.**

1.3.2.5 Lipoteichoic Acids

This substance may function in virulence by inducing the production of tumour necrosis factor (*TNF* **)** and interferon leading to modulation of the immune response (Wicken *et al,* 1963; Tsutsui *et al*, 1991).

1.3.3 Enterococci Infections and Immunity

Infection can occur if other defences of the host are neutralised, avoided or restricted and the pathogens breached mucosal or skin barriers and adhere to the host tissues or cells. However, the studies by Bhakdi *et al* in 1991, had shown that lipoteichoic acids produced by *E.faecalis* stimulate production of Il-β, Il-6 and *TNF*α from the cultured human monocytes. Similarly, Tsutui *et al* in 1991 found that enterococcal lipoteichoic acid was a potent inducer of tumour necrosis factor and interferon. The tissue damage sites of infection could result from activation of complement by the host cell-membrane-associated bacterial lipoteichoic acid (Hummell and Winkelstein, 1986). The interaction between complement , human neutrophils and enterococci has been examined by Harvey *et al*, 1992 who found that it enhanced neutrophil killing of enterococci in the presence of complement and specific rabbit antienterococcal immunoglobin. Arduino *et al* in 1994,

suggested that neutrophil killing depended primarily on complement activation and not specific immuno- globulin. *E.faecalis* secrete small peptides seven to eight amino acid in length called pheromones that promote conjugation transfer of plasmid DNA between strains. These pheromones possess additional role as chemoattractants of neutrophils which induce a respiratory burst (Ember and Hugli, 1989). Enterococci also possess an oxygen-inducible superoxide dismutase which catalyzes conversion of superoxide to hydrogen.

1.4 Drug Resistance in enterococci

Bacterial resistance to antibiotics has been recognised since the first drugs were introduced for clinical use. The consequences of the bacterial resistance to antibiotics must be considered medically and economically in terms of the patients infected with antibiotic resistant pathogens. However, the increasing role of nosocomial infections and the resistance to antibiotics has been documented. The emergence and the spread of *E.faecalis* and *E.faecium* strains with multiple antibiotic resistance continues to be a medical problem (Seetulsingh *et al*, 1996; Huycke *et al*,1998). Enterococci are intrinsically resistant to many commonly-used antibiotics and have the ability to acquire resistance by mutations in the chromosome or through the genes coding resistance from external source via plasmid or transposon (Table 1.3).

1.4.1 Intrinsic Resistance In Enterococci

This type of resistance is also termed "Innate" or " Inherent". The resistance indicates the characteristics present in all or most of the strains of *Enterococcus* species and the genes for resistance appear to reside on chromosomes. The various intrinsic traits expressed by enterococci include resistance to cephalosporins, low- level aminoglycosides and clindamycin (Murray, 1990). Enterococci resistance to β-lactams is a characteristic feature due to low affinity of penicillin-binding protein (Williamson *et al*, 1985). The low-level aminoglycoside resistance involves the reduced uptake of aminoglycoside and enzymatic inactivation of aminoglycoside by chromosome-encoded enzyme (Simjee and Gill, 1997). The *in vitro* and *in vivo* activities of trimethoprim-sulfamethoxazole (TMP/SMX) are controversial. *In vitro*, the media containing thymidine allow many bacteria to escape the inhibition of TMP/SMX by converting thymidine to thymidylate used by the bacteria (Amyes and Smith, 1974; Tofte *et al,*1984).

1.4.2 Genetically acquired Resistance In Enterococci

The three elements involved in acquired resistance in enterococci are : broad-host range plasmids, narrow-host range plasmids and conjugate transposons.

1.4.2.1 Broad-host Range Conjugate Plasmids

The plasmids which can mediate their own transfer are termed conjugate plasmids. Both gram-positive and gram-negative bacteria have the ability to conjugate. In enterococci, the broad-host range conjugate plasmid was originally identified in

E.faecalis (pAMß1) and now occurs across the genus *Enterococcus*. They can also be transferred to other gram-positive bacteria including streptococci, *S.aureus* and *B.subtilis* (Clewell, 1981). However, such plasmids transfer poorly (usually less than 10^{-6} per donor) in broth and require filter mating for efficient transfer. It has been suggested that broad-host range plasmids may be the reason that staphylococci and enterococci share so many resistance genes to gentamicin, erythromycin and penillinase (Murray,1990; Schaberg and Zervos, 1986). The conjugation of broad host-range plasmids is less well understood.

1.4.2.2 Narrow-Host Range or Pheromone Plasmids

The plasmids pAD1 and pAMY1, carry genes for UV light resistance, haemolysin and bacteriocin. In *E.faecalis*, four plasmids (pADI, pCF10, pPDI and pAM373) encoding aggregation substances (AS) have been described (Clewell and Dunny, 2002). However, plasmids pADI and pAMY1 transfer with high frequencies (10^{-3} - 10^{-1} per donor) in broth, higher than the broad-host range conjugate plasmids (Clewell, 1981). Such high transfer is due to chromosomally-encoded oligopeptides called sex pheromones. Thus, the recipient cells secrete the pheromones into the surrounding media, which attracts the donors. Donors then respond by producing aggregation substance (AS) which allows both the donors and the recipients to be in contact and allows the transfer of genetic materials. The recipient then shuts off the system to prevent self-clumping (Dunny *et al*, 1995). Sex pheromone plasmids have also been described in *E.faecium* in which they have been found to be associated with vancomycin resistance (Handwerger *et al*, 1990; Heaton and Handwerger, 1995; Magi *et al*, 2003). However, the role of pheromone plasmids in the spread of resistance genes beyond this species is not clear.

1.4.2.3 The Conjugative Transposons

They are gene sequences or DNA elements that move from the genome of a donor bacterium to the genome of a recipient bacterium by the process of intercellular transposition based on excision, conjugation and re-integration (Clewell and Gawron-Burke, 1986; Clewell *et a*l, 1995; Salyers *et a*l, 1995; Scott and Churchward, 1995; Murray, 1998). For example, the most studied conjugative transposon, Tn*916* carrying the tetracycline resistance determinant *tet*M (Franke and Clewell, 1981), moves via a circular intermediate produced by excision of the integration element from the donor chromosome (Scott *et al*, 1988) which is then transferred from donor to recipient initiating at *ori*T (Scott *et al*, 1994). Once in the recipient, the circular intermediate reforms and integrates into recipient's genome. This process does not generate duplication at the target site into which it inserts. The conjugative transposons have a broad-host range and are found in gram-positive (ie particularly common in enterococi and streptococci) and gram-negative. They play an important role in dissemination of antibiotic resistance in these organisms. Many other conjugative transposons related to Tn*916* have since been described (Salyers *et al,* 1995). On the other hand, transfer of DNA elements in pheromone-inductive plasmids differ from the conjugative transposons in that they are pheromone-responsive conjugation (see section 1.4.2.2) and are restricted to *E.faecalis* (Clewell, 1981; Dunny *et al,* 1995). While conjugation in the broad host-range plasmids is less well understood (Clewell, 1981; Murray, 1998).

Table 1.3 Patterns and Mechanisms of Enterococci Resistance to Antimicrobial Agents

Antibiotic	Mechanisms of Resistanmce
Aminoglycosides	Production of aminoglycoside-modifying enzymes. Ferretti *et al*, 1986; Horodniceanu *et al*,1979; Shaw *et al*,1993 Alteration of the target (leading to impermeability) Moellering and Weinberg,1971
Glycopeptides	Alteration of target site (leading to cell wall Modification). Woodford (1998).
β-lactams	Alteration of target site (ie penicillin-binding proteins) Fontana *et al*, 1996. Production of β-lactamase Murray & Mederski-Samoraj,1983; Murray(1992)
Fluoroquinolones	Alteration of target sites of GyrA & ParC Kanematsu *et al*, 1998; El-Amin *et al*,1999.
Chloramphenicol	Production of acetyl transferase. Triuecuot *et al*, 1993.
Trimethoprim	The use of exogenous folates *in vivo* Grayson *et al*, 1990. Acquisition of dihydrofolate reductase genes Coque *et al*, 1999.

Tetracyclines	Ribosomal protection by *Tet* determinants. Bentorcha *et al*,1992; Charpentier *et al*,1994.
MLS group (Marcolides,	Production of methylating enzymes (*erm*B) Portillo *et al*, 2000; Schmitz *et al*, 2000.
	Efflux of Macrolides (*msr*C) Portillo *et al*, 2000.
Lincosamides and	Inactivation of lincosamides (*lin*B). Bozdogan *et al*, 1999.
Streptogramin)	Inactivation of streptogramin A (*vat*D & *vat*E) Rende-Fournier *et al*, 1993; Werner and Witte, 1999

1.5 The Aminoglycoside Antibiotics

Several aminoglycoside antibiotics have been introduced into use since the discovery of streptomycin in 1943 from *Streptomyces griseus*. Kanamycin was introduced in 1957 and became the aminoglycoside drug of the choice until 1963 when the gentamicin was discovered and used for the treatment of gram-negative bacillary infections. Subsequently, amikacin (1976), tobramycin and netilmicin (1983) were developed for clinical use (Zembower *et al*,1998). Aminoglycosides have the potential of nephro-toxicity and their use has been enhanced by dose management by monitoring both serum levels and renal function. Aminoglycosides have less allergic reactions and other adverse effects. However, despite the advent of new β-lactams and fluoroquinolones, the aminoglycosides remain useful agents against serious infections such as infective endocarditis and bacteraemia caused by the same strains of *Enterococcus* species. Structurally, aminoglycosides consist of a

six-membered amino group which contain an aminocylitol ring that is linked to two sugars (Fig 1.2). The aminocylitol ring of streptomycin is a streptidine and other aminoglycosides consist of 2-deoxystreptamine (Fig 1.2). The 2-deoxystreptamine is further subdivided on the bases of their 4,5 and 4,6 of their substitutuents on 2-deoxystreptamine ring (Courvalin and Carlier,1981). The aminoglycosides are highly polar, water soluble polycation and are generally stable to heat and pH change ranging from 5 to 8.

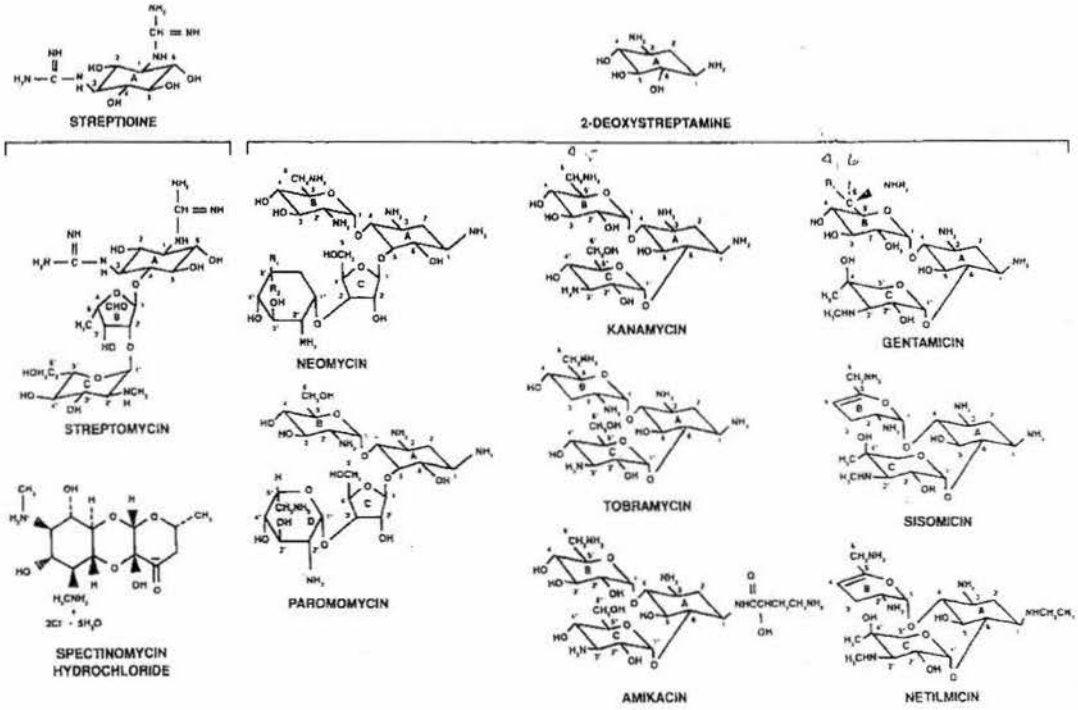
1.5.1 Mechanism of Action in Aminoglycosides

Aminoglycosides inhibit protein synthesis by binding irreversibly to the bacterial 30s ribosome and thereby interrupting the flow of genetic information. Streptomycin's sensitivity to 30s ribosome subunit has been shown to be determined by binding to a single protein S12. The uptake of aminoglycosides for various organisms especially gram-positive cocci is facilitated by the presence of inhibitor of synthesis of the bacterial cell wall such as β-lactam antibiotics and glycopeptides (Moellering *et al*, 1971). The active uptake of aminoglycoside across the plasma membrane takes place in three stages. The first phase uptake is the rapid energy-independent binding to cell surface(EIP) (Nakae and Nakae, 1982). The second phase involves electrical potential across the plasma membrane generated by aerobic metabolism (EDP1) (Byran and Kwan, 1983). The third phase is triggered by interaction of aminoglycoside with ribosome (EDP11) (Busse *et al,* 1992). However, under anaerobic conditions, the rate of uptake of aminoglycoside into the cell is diminished and therefore bacteria become relatively resistant to aminoglycoside.

1.5.2 Spectrum of Antimicrobial Activity In Aminoglycosides

Antibacterial activity of aminoglycosides is directed against a broad spectrum of aerobic and facultative anaerobic gram-negative bacilli and many staphylococci and certain mycobacteria. However, gentamicin is the choice for the treatment of serious hospital-acquired infections caused by enterobacteriaceae and *P.aeruginosa*. In addition, gentamicin can be combined with β-lactams or glycopeptides to provide synergy in the treatment of serious infections such as infective endocarditis and bacteraemia caused by enterococci, staphylococci and viridans streptococci.

Fig 1.2 The Structures of Aminoglycosides



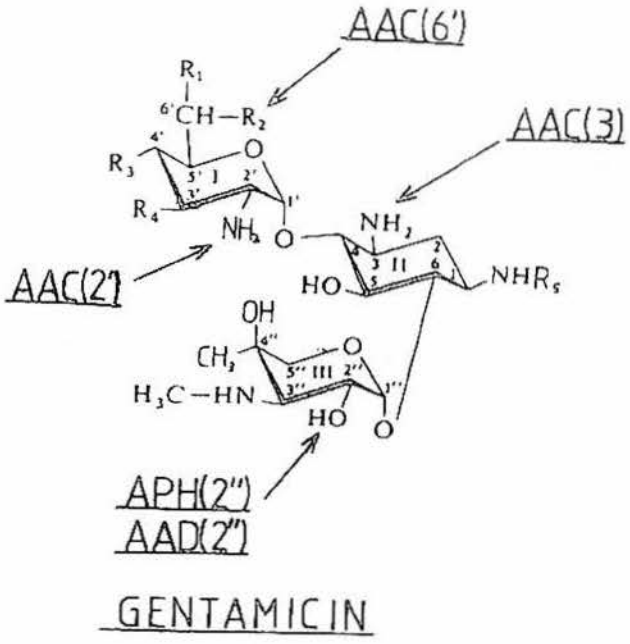
Adapted from Gilbert D.N, 2000

1.6 Resistance of Enterococci to Aminoglycosides

The aminoglycosides act by interfering with protein synthesis by binding to 16S rRNA of the 30s ribosomal subunit (Kotra *et al*, 2000; Mingeot-Leclercq *et al*, 1999). Enterococci possess intrinsic low-level of resistance to aminoglycoside which limit transport of the drug across the cell membrane or due to mutation in chromosome reducing the uptake of aminoglycoside (Lefort *et al*, 2000). The low-level resistance of enterococci against gentamicin, usually, has MIC range of 8-64mg/l (Murray, 1990; Bantar *et al*, 1993). Aminoglycosides are not so effective against enterococci when used alone. However, the addition of an agent that interferes with cell-wall synthesis such as amoxicillin or vancomycin increases the uptake of aminoglycoside and enhances the killing of the *Enterococcus* (Moellering and Weinberg, 1971). Enterococci have acquired aminoglycoside resistance genes that encode various aminoglycoside-modifying enzymes resulting in a very high resistance to aminoglycoside, with MICs usually >2000mg/l (Murray, 1990), thereby eliminating the synergistic killing effect of the combined aminoglycoside with β-lactam or glycopeptide. The most clinically important of AME is the bifunctional gene *aac(6')-le-aph(2")-la* (Ferretti *et al*, 1986) that possesses both acetylating and phoshorylating activity (Azucena *et al*, 1997; Ferretti *et al*, 1986). The gene appears to have occurred from a fusion of two ancestral genes which mediates resistance to a broad range of aminoglycosides including gentamicin, tobramycin, kanamycin and dibekacin. This gene has also been detected in *S.aureus* and several *Streptococcus* species (Galimand *et al*, 1999). It has been reported recently of the new aminoglycoside resistance genes such as *aph(2")-lc* (Chow *et al*, 1997), *aph(2")-ld* (Tsai *et al*, 1998) and *aph(2")-lb* (Kao *et al*, 2000) which can confer the resistance to gentamicin by enterococci just like *aac(6')-le-aph(2")-la* gene. The *aph(2")-lc* gene

encodes an aminoglycoside phosphotransferase that mediates clinical resistance to gentamycin, tobramycin, kanamycin and dibekacin but not amikacin or netilmacin (Chow *et al*,1997). Although the gene was first detected in a conjugative plasmid from *E.gallinarum*, it has now been detected in both *E.faecalis* and *E.faecium* with MIC range of 256-384mg/l. The *aph(2")-ld* gene encodes an aminoglycoside phosphotransferase that mediates high-level resistance to gentamicin, tobramycin, kanamycin, netilmicin and dibekacin (Tsai *et al,*1998). The gene initially cloned from *E.casseliflavous* and now detected in *E.faecium* clinical isolate with MIC >2000mg/l. The *aph(2")-lb* gene mediates high-level resistance to gentamicin, tobramycin, kaamycin, netilmicin and dibekacin. It has been detected in VRE *E.faecium* clinical isolate (Kao *et al*, 2000). Resistance of enterococci to streptomycin is based on change(s) in the target site of protein S12 of the 30s ribosomal subunit that results in decreases binding of streptomycin (Eliopoulous *et al*, 1984). The resistance to aminoglycoside by enterococci associated with a change in the ribosome has not been reported other than in streptomycin. The *ant(6')-la* and *ant(3")-la* genes which encode streptomycin nucleotidyltransferase [*ANT(6')-la* and *ANT(3")-la* respectively] can confer hgh-level streptomycin resistance in enterococci with MICs range of 4000-16000mg/l (Clark *et al*, 1999; Eliopoulos *et al*, 1984)

Fig 1.3 The Chemical Structures Of Gentamicin



AAD=Aminoglycoside adenyltransferase same as ANT=Adenyltransferase

1.6.1 Molecular Basis of Aminoglycoside *AAC(6')-APH(2")* (AMEs)

The molecular basis of resistance is the covalent modification of the drugs. Specifically, *AAC(6')-APH(2")* which harbours both acetyl COA-dependent N-acetyltransferase and ATP-dependent O-phosphotransferase activities (Ferretti *et al*, 1986) (Fig 1.3). This indicates that the gene has arisen by fusion of *aac* and *aph* genes. The amino-acid sequence alignment-analysis has revealed homology to other

aminoglycoside N-acetyltransferases in the amino-terminal region and aminoglycoside kinases in the carboxy-terminal truncated enzymes (Ferretti *et al*,1986). This bifunctional enzyme is very effective at conferring resistance to virtually all aminoglycosides that incorporate a 2-deoxystreptamine ring including the clinically important antibiotics such as gentamicin C, tobramycin and amikacin.

Fig 1.4 Biochemical Mechanisms of Aminoglycoside Modification

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AMINOGLYCOSIDE + ATP--***APH****-->AMINOGLYCOSIDE-**O-P-OH** + ADP
OH

AMINOGLYCOSIDE + ATP-***AAD****→AMINOGLYCOSIDE-**O**-ADENOSINE +PPi

AMINOGLYCOSIDE + ATP-***AAC****->AMINOGLYCOSIDE-**NH**-CO-CH3+COA-SH

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*Modification of aminoglycosides by *APH*, *AAD* & *AAC*. *APH*=Phosphotransferases, *AAD* or ANT = Adenyltransferases & *AAC*= Acetyltransferases.

1.7 Quinopristin (S_B)-Dalfopristin (S_A) Resistance in Enterococci

The combination of quinupristin and dalfopristin called "Synercid" is used in the treatment of multidrug-resistance *E.faecium* infections. Dalfopristin is a streptogramin A while quinupristin is a streptogramin B. The two compounds act synergistically by streptogramin A binding to 50s ribosomal subunit causing a conformational change in the peptidyltransferase domain of the ribosome which increases affinity of streptogramin B to its target site resulting in the inhibition of protein synthesis (Vannuffel *et al*, 1994). All *E.faecalis* isolates are intrinsically resistance to streptomycin A compounds which make them resistance to synercid. However, resistance to synercid by *E.faecium* can be due to a single gene (*vat*D) that mediates resistance to streptogramin A or to synercid

(Q/D) resistance. The *vat*D gene, formerly *sat*A, cloned from *E.faecium* encodes an amino acid sequence that is closely related to acetyltransferase which mediates resistance to streptogramin A compounds (Rende-Fournier *et al*, 1993). *Vat*E (formerly *sat*G) gene encodes a putative acetyltransferase and appears to be prevalent in *E.faecium* (Werner and Witte, 1999). Plasmid mediated genes (*vat*D & *vat*E) are related to acetyltransferase genes *vat* (7), *vat* (2) and *vat* (C) reported in staphylococcal resistant to quinupristin B (Cocito *et al,* 1997). Thal and Zervos, 1999 reported *E.faecium* resistance to synercid with MIC of 32mg/l. The same year, Arum, this thesis, (unreported)-found *E.faecium* clinical isolates resistant to synercid with MIC of 64mg/l. Although, it has not been studied much in *E.faecium*, the active efflux associated with ATP-binding cassette transporters were reported to confer resistance to streptogramin A (Allignet and Solh, 1997).

1.8 Quinolones Resistance In Enterococci

The first fluoroquinolone widely used in the clinical treatment was ciprofloxacin and developed for use against gram-negative bacterial infections. However, the activity of ciprofloxacin against the enterococci is moderate. Quinolone resistance is common in the clinical enterococcal isolates. The newer fluoroquinolones such as moxifloxacin has slightly enhanced *in vitro* activity against enterococci although isolates resistant to ciprofloxacin are also resistant to moxifloxacin. Quinolones inhibit bacteria by interacting with type 11 topoisomerase (*gyr*A) subunit and topoisomerase 1V (*par*C) subunit which are responsible for bacterial DNA replication. In *E.coli*, quinolones bind to a complex of DNA and DNA gyrase and not DNA alone (Shen *et al*, 1989). The primary target for quinolones against gram-negative bacteria such as *E.coli* is *gyr*A subunit of DNA gyrase while *par*C subunit of topoisomerase 1V is the primary target for quinolones against gram-positive bacteria such as *S.aureus* and *E.faecalis* (Ng *et al*,

1996). DNA gyrase is composed of two A and two B subunits (*gyr*A and *gyr*B) (Wang, 1996). Topoisomerase 1V is composed of two subunits (*par*C and *par*E) which are homologous to *gyr*A and *gyr*B (Kato *et al*, 1990).The studies by Kanamatu *et al*, 1998 found an isolate with an alteration only in topoisomerase 1V and none in *gyr*A. Brisse *et al*, 1999 found some isolates of *E.faecium* with alterations only in *par*C and not in *gyr*A suggesting that *par*C is the primary target of ciprofloxacin in *E.faecium*. However, other studies have shown that either *gyr*A or *par*C can be the primary target depending on the structure of fluoroquinolones (Pan and Fisher, 1997). Quinolone resistance has not been well studied in enterococci in comparison to that in staphylococci and pneumococci. However, mutations in the *par*C gene resulting in substitution of one or two amino acids corresponding to *E.coli gyr*A quinolone resistance determining region(QRDR) could be the first step in quinolone resistance. An additional mutation in QRDR in enterococcal *gyr*A may then follow resulting in a high-level resistance to quinolone. No *gyr*B or *par*E resistance due to mutations have been reported in enterococci (Hooper, 2000). The demonstration of the importance of *gyr*A mutation contributing to quinolone resistance was done by showing that *gyr*A subunit purified from a quinolone resistant *E.faecalis* combined with wild type *gyr*B subunit conferred quinolone resistance (Nakansishi *et al*, 1991).

1.9 Penicillin Resistance in Enterococci

The target sites for penicillin and other ß-lactam antibiotics are the final cross-linking reaction that gives the bacterial cell-wall rigidity and penicillin-binding proteins (PBPs). Penicillin can also facilitate the access of compounds acting on internal targets, potentiating inhibition or killing, as in classical synergy with aminoglycosides. Enterococci are relatively resistant to β-lactams. *E.faecium* being inherently more

resistant to penicillin (MIC 16-32mg/l) than *E.faecalis* (MICs 2-4mg/l)(Gordon *et al*, 1992; Moellering *et al*, 1979). The intrinsic resistance as well as high levels of resistance to penicillin has been associated with overproduction of low-affinity to penicillin-binding protein 5 (*PBP5*) and the production of *β*-lactamase (Murray, 1992). Also amino acid substituted within the *PBP* causes further decrease in affinity in penicillin associated with a deletion upstream of the *PBP5* gene (Fontana et al, 1996), *β*-lactamase production in *E.faecalis* was first reported in 1983 (Murray & Mederki, 1983). This enzyme is produced by enterococci constitutively instead of inducible found in staphalococci. However, it has been reported that non ß-lactamase producing *E.faecium* also confer high-level penicillin resistance associated with the overproduction of low-affinity PBP5 (Bush *et al,* 1989; Sapico *et al*, 1989). *In vitro*, enterococcal *β*-lactamase has greater activity against penicillin and ampicillin but little or no activity against most cephalosporins and imipenem. *β*-lactamase is encoded on transferable plasmids in a number of *E.faecalis* (Markowitz *et al,* 1991). *β*-lactamase production in enterococci has not been isolated in Europe but only in USA.

1.10 Glycopeptide Resistance in enterococci

The activity of glycopeptide antibiotics is restricted to gram-positive organisms whether anaerobes or aerobes because antibiotics cannot penetrate the outer membrane of gram-negative organisms due to being large polar molecules. Although, both vancomycin and teicoplanin are glycopeptide antibiotics, their spectrum activity is not identical (Greenwood, 1988). Teicoplanin is more active against gram-positve anaerobes and streptococci than vancomycin; but vancomycin is more active against coagulase-negative staphylococci. All glycopeptide antibiotics inhibit the latter stages of cell wall synthesis

by forming complexes with peptidoglycan precursors (Arthur *et al*, 1996b). It occurs by binding the antibiotics to D-Ala-D-Ala-terminating peptide stems within nascent peptidoglycan which is believed to inhibit cell wall synthesis through inhibition of the transpeptidase and carboxypeptidase steps of cell wall synthesis. Glycopeptide-resistant enterococci was first detected in UK and in France in 1986 (Leclercq *et al*, 1988; Uttley *et al,* 1988). In the same year (1986), vancomycin resistance in enterococci was described in USA in broiler chickens and pigs in the reports on tetracycline feeding trials (Molitoris *et al*, 1986). Various types of vancomycin-resistant enterococci (VRE) have been characterised on phenotypic and genotypic bases (Perichon *et al,* 1997; Fines *et al,* 1999; Arthur and Courvalin,1993 Mckessar *et al*, 2000). There are five recognized phenotypes of vancomycin resistance: *van*A, *van*B, *van*C, *van*D, and *van*E. However, Mckessar *et al*, in 2000 isolated and sequenced putative vancomycin resistance that they designated as *van*G. Four of these (ie *van*A, *van*B, *van*D and *van*E) have the acquired resistance mechanisms and *van*C is an intrinsic resistance mechanism. The *van*A and *van*B types were described mainly in *E.faecalis* and *E.faecium*. *Van*A resistant strains possess inducible, high-level-resistance to vancomycin (MIC >64mg/l) and teicoplanin (MICs >16mg/l) (Arthur and Courvalin, 1993). The genes for *van*A resistance phenotyope are located on transposable element *Tn*1546. They encode 7 polypeptides which involve in resistance to both vancomycin and teicoplanin (Evers and Courvalin, 1996). However, mutants derived from *van*B strains may exhibit resistance to teicoplanin and are thus phenotypically indistinguishable from *van*A (Hayden *et al*, 1993). *Van*C resistance phenotypes are characterized by low-level resistance to vancomycin but are susceptible to teicoplanin (Arthur *et al*, 1996). The *van*A gene and other genes are involved in regulation and expression of vancomycin resistance (*van*R, *van*S, *van*H, *van*X, *van*Y and *van*Z). They are all located on Tn*1546* of *E.faecium* (

Arthur *et al*, 1993). The presence of variable amount of D-Ala-D-Ala relative to D-Ala-D-Ser could account for variable levels of vancomycin resistance observed among the isolates of VRE carrying the *van*C phenotype (Murray,1997). *Van*A, *van*B & *van*D have altered substrate specificity preferentially producing D-Ala-D-Lac precursors while *van*C ligase produces D-Ala-D-Ser depeptide. The *van*D phenotype resistance shows 67% identity with *van*A and *van*B (Ostrowsky *et al*, 1999). The *van*E phenotype resistance has been described in *E.faecalis* recently. It is resistant to low-levels of vancomycin (MIC 16mg/l) and teicoplanin (MIC 0.5mg/l) (Fines *et al,* 1999).

1.11 Oxazolidinone resistance in Enterococci

The oxazolidinones are a new class of antimicrobial agents developed for use against multidrug-resistant gram-positive bacteria. Linezolid was the first compound to be approved for clinical use in USA. Oxazolidinone acts by inhibiting bacterial translation in the initiation phase of protein synthesis thus preventing formation of N-formyl-metionyl-tRNA-ribosome-ternary complex (Swaney *et al,* 1998). Resistance to linezolid by *E.faecium* has been reported by Gonzales *et al*, 2001.

1.12 The *AAC(6')-APH(2")* Transposon Tn*5281*

In 1990, Holdel-Christian and Murray identified a composite transposon designated *Tn*5281 responsible for conferring high-level gentamicin resistance in *E.faecalis* by the virtue of the 2kb bifunctional gene (*aac6'-aph2"*) flanked by 1.35kb IS element designated IS256 (Hodel-Christian and Murray,1990). Rice *et al* in 1995, reported a 26kb composite transposon in *E.faecalis* designated *Tn*5384 conferring high-level gentamicin and erythromycin resistance. Two of IS256 which flank the *aac6'-aph2"* gene conferring the HLGR phenotype appear to be conserved in all enterococcal species but differ in the flanking regions immediately adjacent to the fused gene.

1.12.1 The Origin of AME Resistance Genes

Aminoglycoside-modifying enzyme genes in enterococci appear to have originated from staphylococci because hybridization studies, using *Enterococcus*-derived probes for AMEs (*ant6*', *aph3*', *aac6'-aph2*" and *ant4'*), demonstrated an extremely high degree of homology between those of staphylococci and enterococci AME genes (Ounissi *et al,*1990). Similarities were also observed between staphylococcal HLGR transposon for Tn*4001* and Tn*4031* and the enterococcal HLGR transposon Tn *5281* (Hodel-Christian and Murray, 1991). However, how the AME genes found their way into enterococci remains unclear but is likely due to inter-genus transfer.

1.12.2 Regulation of Aminoglycoside Resistance Genes

The aminoglycoside resistance genes in general, do not appear to be regulated. The transcription of these genes is apparently constitutive and provides constant protection against the presence of aminoglycosides. However, the exception to this generalization is that, the expression of the chromosomal *aac(6')-lc* genes of *S.marcescens* and the gene of *Providencia stuartii* appear to be tightly regulated (Rather *et a*l, 1992).

1.13 The impact of Aminoglycoside Resistance in Enterococci

The high–level aminoglycoside resistance among enterococci is increasingly being reported world-wide (Huycke *et al,* 1998). Nicole van den Braak *et al* in 1999, reported the prevalence of HLGR enterococci in the patients with blood infections with significant increase from 14% in 1991 to 31% in 1997 at the Dutch University Hospital. An increase in HLGRE isolates from 17% to 60% in two Dublin

Hospitals from 1991 to 1994 (Lavery *et al,* 1997) has been reported.

1.13.1 Aminoglycoside-Resistant Enterococci Mortality Rates

The mortality among patients whose bacteraemia was due to HLGRE infections in Chicago Memorial Hospital was found to be higher than those caused by susceptible enterococci (Noskin *et al*, 1995). All four bacteraemia patients who had *E.faecium* infections with high levels of both vancomycin and gentamicin resistance died (Noskin *et al,* 1995).

1.14 The Impact of Glycopeptide Resistance in Enterococci

The percentage of vancomycin-resistant enterococci increased from 0.3% to 7.9% in USA between 1989 to 1993 (MMWR, 1993). The increase has been most profound in Intensive Care Units where a 34-fold increase in the incidence has been noted (MMWR, 1993)

1.14.1 Glycopeptide-Resistant Enterococci Mortality Rates

The mortality associated with bacteraemia caused by VRE has been reported in USA to be 37% as compared with 16% for susceptible ones (MMWR,1993). Similar trends could be expected to occur in UK although I could not obtain any data to support this view.

1.15 Treatment of Infections caused by HLGRE and GRE

1.15.1 Treatment of Infections caused by HLGRE

Enterococci with high-level resistance to gentamicin are usually resistant to all other aminoglycosides but occasionally are susceptible to streptomycin (Spiegel and Huycke, 1989). However, cell-wall active agents such as ampicillin, penicillin or

vancomycin may be used alone in an attempt to cure certain enterococcal infections such as urinary tract infections. But the cell-wall antibiotics must be combined with anaminoglycoside in the treatment of bacteraemia and infective endocarditis (Shlaes *et al*, 1981; Nackamkin *et al*, 1988; Maki and Agger, 1988).

1.15.2 Treatment of Infections caused by GRE

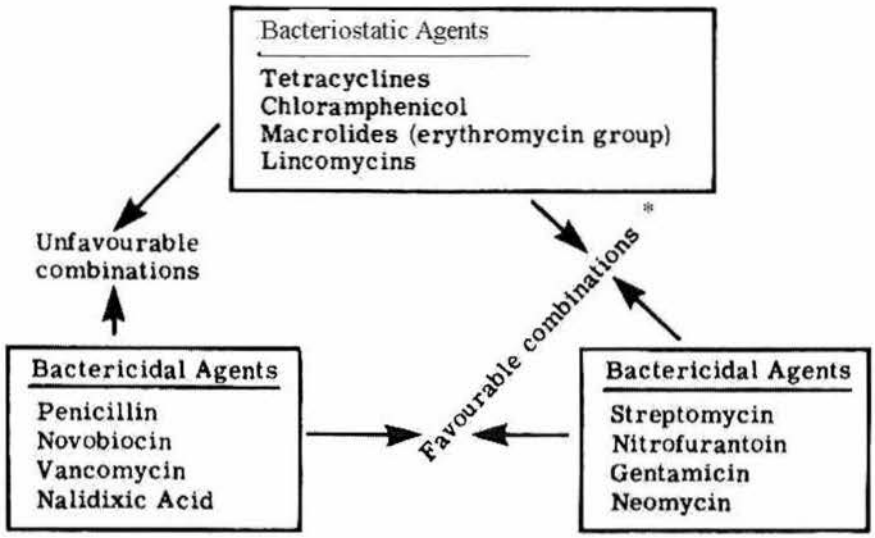
The treatment of the infections caused by VRE especially *E.faecium* is extremely difficult because the organisms are resistant to multiple antibiotics (Cetinkaya *et al*, 2000). However, successful treatment depends on laboratory antibiotic test against VRE.

1.16 Antimicrobial Synergism

The increased incidence of resistant strains of microorganisms, following the use and misuse of antimicrobial drugs on a large scale, had led to the administration of combinations of drugs (Jawetz *et al,* 1955; Moellering *et al*, 1971; Simmons, 1975; Berenbaum,1978; Rahal, 1978; Lambert *et al*, 2003). The most appealing reason to utilize antimicrobial combinations is to produce enhanced (synergistic) antimicrobial activity. In many instances, this has been demonstrated by *in vitro* tests. The term synergy has been defined as the effect obtained from the use of A combination of antibiotics having greater than the sum of the effects of each of the component agents acting alone (Dowling, 1957; Mounton,1975; Berenbaum, 1978; Lambert *et al* 2003). On the other hand, if the effects of each of the component agents is greater than the effect of the combined agents, then it would indicate antagonism. Usually antagonism occurs between predominantly bacteriostatic agents

and bactericidal agents (Fig 1.5). Antibiotic combinations are widely prescribed for the following reasons: to treat mixed infections, to prevent or delay the appearance of resistant strains, to treat serious infection such as meningitis before the results of laboratory tests are known and in the use of non-toxic combined drugs instead of a single toxic drug (Rahal,1978) and to produce synergistic activity. The combination of a cell-wall agent such as penicillin or vancomycin with an aminoglycoside is widely accepted in the treatment of enterococcal endocarditis (Rahal, 1978). Antagonism has been observed between penicillin and tetracycline in pneumococcal meningitis (Lepper and Dowling, 1951; Mounton,1975). Shlaes *et al*, (1991) demonstrated synergy between penicillin plus vancomycin plus gentamicin against enterococcal clinical isolates. Vancomycin combined with aminoglycoside has shown synergistic activity against enterococci both *in vivo* and *in vitro* (Westenfelder *et al*, 1973). However, the presence of high-level gentamicin- resistant enterococci renders all the aminoglycoside antibiotics inactive and also the presence of VRE with multiple antibiotic resistance renders most of the antibiotics, currently used at present inactive. Therefore, new drug(s) or combinations must be found to cure the serious infections caused by the highly drug-resistant enterococci.

Fig 1.5 Predicting the Effect of Combinations of Antibacterial drugs



Adapted from Mounton, 1975. *= Most or all Bacteriostatic drugs are no longer favourable combinations with Bactericidal Agents of Streptomycin-----→Neomycin

1.17 Epidemiology of HLGRE and GRE

1.17.1 The Epidemiology of GRE

Nosocomial outbreaks of vancomycin- resistant strains of enterococci have been reported throughout the USA and other parts of the world (Swartz, 1994). The outbreaks of GRE infection mainly occur in renal, haematological and intensive care units (Woodford,1998) of the hospital. A predominant strain of GRE may be responsible for such outbreaks. However, strains from different hospitals may not be the same and the sporadic strains tend to be unique when observed by PFGE technique (Woodford, 1998). In USA, most hospitals usually report a predominant single strain (Murray, 1997). In UK, the GRE isolated in hospitals across the country appears to suggest that they emerged independently at different centres based on their endemicity in the community and subsequent selection due to antibiotic therapy in the hospital settings (Woodford, 1998). Enterococci appear to have

evolved perfectly to spread and transmit vancomycin-resistance genes and other resistance genes within both hospital and community settings (French, 1998). There is a real danger that *van*A resistance will spread from enterococci to staphylococci and pneumococci in future (French, 1998).

1.17.2 The Epidemiology of HLGRE

The high-level gentamicin-resistant (HLGR; MIC >1000mg/l) strains of *Enterococcus faecalis* were first reported in France in 1979 (Horodniceanu *et al,* 1979) and have subsequently become disseminated worldwide (Murray, 1990). In *E.faecium*, HLGR appeared in USA in 1986 (Eliopoulos *et al.* 1988). The emergence of such strains (ie *E.faecium*) had been predicted previously from *in vitro* transfers of gentamicin resistance (Gm^r) plasmids from *E.faecalis* to *E.faecium* (Chen and Williams,1985). Casetta *et al* in 1998 detected 20 strains of *E.faecalis* carrying Gm^r plasmids (pIP655) with Tn*4001*-truncated elements. However, there are now a number of reports of the isolation of HLGR *E.faecium* in several countries including UK, Ireland, Singapore and Australia (Bendall *et al*, 1991; Lin and Tan, 1991; Wade *et al*, 1991, Woodford *et al,*1991; Woodford *et al*, 1993). In Greece, high-level gentamicin resistance is encountered in 15.4% of nosocomial *E.faecalis* and 11% of *E.faecium* (Paparaskevas et al , 2000). In both *E.faecalis* and *E.faecium*, HLGR is often disseminated by plasmid or transposon. In five Nordic hospitals Simonsen *et al*, 2003, found the prevalence of HLGR to be low at Reykjavik (1.1%), Tromsø (3.6%) and Aarhus (1.2%). However, the prevalence of HLGR of *E.faecalis* in Uppsala was found to be 27.6%; while in Bergen it was 15.7%. In *E.faecium* in Aarthus, HLGR was found to be 58.8% in comparison to the situation reported in other countries.

Aims of This Thesis

1. To establish whether the clinical isolates are β-lactamase producers or not.
2. To characterise the differences in antimicrobial susceptibility among the isolates.
3. To characterise the pattern of synergy of the combination of antibiotics against *E.faecalis* and *E.faecium*.
4. To establish the patterns of resistance among the clinical isolates of *E.faecalis* and *E.faecium*.
5. To characterise the haemolytic patterns of *E.faecalis* and *E.faecium* on Horse- blood media.
6. To establish the biochemical differences among the *E.faecalis* and *E.faecium* strains

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial isolates

Clinical isolates of gentamicin-resistant and vancomycin-resistant enterococci were obtained from the collection of Dr. Ngozi Elumogo,Clinical Bacteriology, Edinburgh University Medical School. Three enterococcal isolates of *van*B were kindly donated by Dr. Karen McGregor of the Department of Biological Sciences, University of Dundee. The strains used as reference or control organisms indicated in Table 2.1 were kindly donated by Dr. Alan Brown of the Dept of Medical Microbiology, Edinburgh University Medical School. Strains NCTC 50192 (39R861), NCTC 50193 (V517) and NCTC 50265 were obtained from the Central Public Health Laboratory, Colindale.

Table 2.1 : Reference Bacterial Strains

Bacterial Strains	**Resistance**
E.faecium NCTC12202	***Van*A Phenotype**
E.faecalis ATCC 51299	***Van*B Phenotype**
E.faecalis NCTC 12697	Sensitive
E.faecalis NCTC 29212	Gentamicin Resistant

E.faecium	NCTC 7171	Sensitive
E.faecium	788/5/95 (Clinical)	*Van*A Phenotype

2.1.2 Chemical Reagents

Unless otherwise stated, all chemicals used were provided by Sigma-Aldrich Coy Ltd, Poole, Dorset, UK

2.1.3 Media

Unless otherwise stated, all media were obtained from Oxoid, Basingstoke, UK. Media used were Brain Heart Infusion (BHI) Agar/Broth, Isosensitest Agar (ISTA), and Mueller-Hinton Agar (MHA) and prepared according to the manufacturer's instructions. In most cases, all cultures were prepared in BHI media unless otherwise stated and sterilized by autoclaving at 121°C at 15 psi for 15 minutes. Beads media were obtained from Microbank, PROLAB Diagnostics, UK.

2.1.4 Antimicrobial Agents

Antimicrobial agents listed in Table 2.2 were used in this study. All were stored at 4°C and fresh stock solution prepared as required in sterile deionized water.

Table 2.2 Antimicrobial agents

Antibiotic	Supplier
Amoxicillin	CP Pharmaceuticals Ltd, Wrexham, UK
Augmentin	Beecham Research, Hertfordshire, UK
Vancomycin	Eli Lilly and Co Ltd, Basingstoke, UK
Teicoplanin	Marion Merrel, Uxbridge, UK
Piperacillin	Generic (UK) Ltd, Hertfordshire, UK
Piperacillin/Tazobactam (Tazocin)	Cyanamid of Great Britain Ltd, Hants, UK
Gentamicin	David Bull Labs, Warwick, UK
Ciprofloxacin	Bayer AG, Germany
Moxifloxacin	Bayer AG, Germany
Synercid	Rhône-Poulene Rover, France
Linezolid	Pharmacia and Upjohn Ltd (UK)

2.1.5 Oligonucleotide primers

All the primers used were synthesized by Oswel DNA Service Ltd, Southampton and MWG-Biotech AG, UK. Primers for sequencing were HPLC purified.

2.2 METHODS

2.2.1 Storage of Clinical Isolates

All the isolates including the controls were first subcultured onto BHIA and incubated at 37°C for 18-24 hours. After overnight incubation, a loopful of growth from each plate was placed into each medium containing beads, labelled with each isolate number and stored at -70°C for the future use. Identification of the isolates was based on conventional test schemes recommended by Facklam and Collins, (1989) and Ruoff *et al,* (1990) and confirmed with the aid of API 20 Streps (bioMeriux, Basingstoke, UK).

2.2.2 Identification of *E.faecalis* and *E.faecium* by PCR

The differences between *E.faecalis* and *E.faecium* were established by PCR based on ddl $_{E.faecalis}$ and ddl $_{E.faecium}$ with the primers described by Dutka-Malen *et al* in 1995 as indicated in Table 2.3.

Table 2.3 : Primers for ddl $_{E.faecalis}$ and ddl $_{E.faecium}$

Amplified gene	Primer	Sequence 5' – 3'	Size of amplicon	Position
ddl $_{E.faecalis}$	E_1	ATCAAGTACAGTTAGTCTT	941bp	98-116
	E_2	ACGATTCAAAGCTAACTG		1038-1021
ddl $_{E.faecium}$	F_1	GCAAGGCTTCTTAGAGA	550bp	N/A
	F_2	CATCGTGTAAGCTAACTTC		N/A

1 = Forward Primer, 2 = Reverse Primer, ddl = D-Alanyl-D-Ligase.
N/A = Not Avialable.

2.2.3 Minimum Inhibitory Concentrations (MICs) Determination By Agar Dilution Method.

Minimum inhibitory concentrations of antibiotics were determined by the agar method based on the guideline of the National Committee for Clinical Laboratory Standards [NCCLS, (1997)], on BHIA blood plates. One ml of each of serial doubling dilutions of test antibiotics from the fresh stock was added to 18 ml volume of molted BHIA (Oxoid) cooled at 50°C and adding 1 ml of defribrinated horse blood (E & O Laboratory, UK) and mixed before pouring into each Petri dish. The control plate without antibiotic was also prepared. The plates were allowed to dry for 2 hours and bacterial inocula were prepared by appropriate dilutions of overnight BHI broth cultures subcultured and incubated at 37°C from the isolates stored at -70°C and applied to antibiotic-containing plates with a 36 prong inoculating device (Denley A400) yielding final inocula of approximately 10^4 CFU per spot. The plates were allowed to dry and incubated at 37°C for 18-24 hours. The presence or absence of growth on each plate was noted and recorded for each antibiotic dilution. The lowest concentration of antibiotic which inhibited growth was designated the MIC. The concentration of antibiotic required to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of isolates were determined when required.

2.2.4 β-lactamase Test

The confirmation of presence or absence of β-lactamase production by clinical isolates was done by using nitrocefin-based chromogenic cephalosporin method first described by O'Callaghan *et al*, 1972. It involved transferring 50µl of nitrocefin solution to each well of a microtitre tray. A dense suspension of several colonies from the overnight growth of each isolate was made in each small volume of saline.

Fifty microliters of each suspension was then mixed with solution of nitrocefin and incubated at 37°C for 30 minutes. Absence or presence of colour change from yellow to red within 30 minutes by the isolates was recorded.

2.2.5 Synergy/Antagonism Testing

2.2.5.1 By Agar dilution method

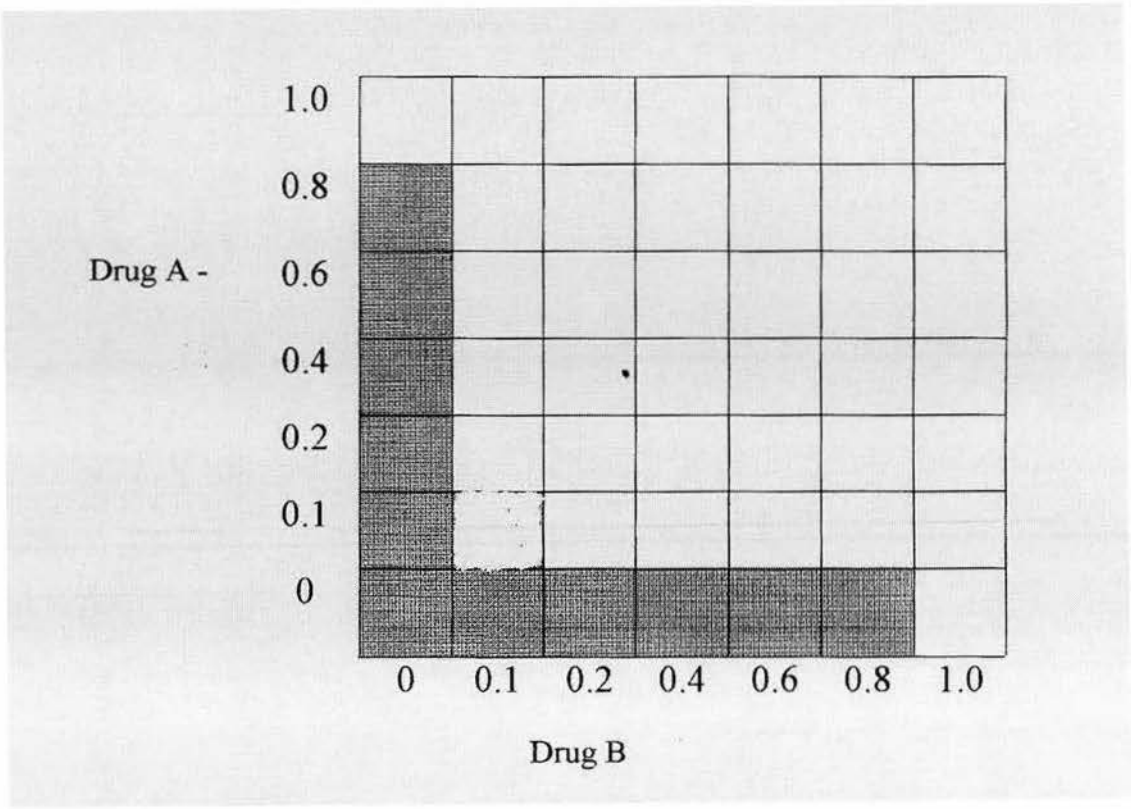
MICs for the combined antimicrobial activity to demonstrate synergy/antagonism were performed using fresh stock solutions. In each case, one ml of each serial two-fold dilution of one antimicrobial agent (range 256mg/l-0.25mg/l) was mixed with one ml of fixed concentration of another antimicrobial agent (ie 4 mg/l) in 18ml of molted agar cooled at 50°C and adding defribinated horse blood and poured into each Petri dish. The control plate without antibiotic was also prepared. The plates were allowed to dry for 2-3 hours before inoculating and incubating isolates as indicated in section 2.2.3 by agar method. The absence or presence of the growth was recorded for each plate.

2.2.5.2 By Checkerboard agar dilution method

The MIC for each antibiotic was measured for each isolate by doubling agar dilution and then the known MIC for each drug was fine-tuned by re-measuring in an arithmetical progression as in Fig 2.1(shaded areas indicated single antibiotic A or B concentrations as controls). The MIC was taken as fractional inhibitory concentration (FIC) of 1.0 for antibiotic. Agar plates each containing a mixture of both drugs were made up as shown in unshaded areas (Row/Column) in Fig 2.1 . The plates were inoculated with isolates using a multiple inoculating device (Denley

A400) yielding final inocula of approximately 10^4 cfu per spot. The plates were examined and recorded for absence or presence of growth after 24 hours of incubation at 37°C.

Fig 2.1 Diagram of a typical Checkerboard



2.2.5.3 By Killing Curves technique

The rate of killing of the clinical isolates by antimicrobial agents alone and by combination with another antimicrobial agent was determined for the selected strains of enterococci (17 *E.faecium* and 22 *E.faecalis*) based on their MICs as well as limited financial resources available to complete the remaining strains by performing colony counts. The following concentrations were used: gentamicin (64mg/l), amoxicillin (8,16 and 32mg/l),teicoplanin (2mg/l), vancomycin (4 and 8mg/l), synercid (2,8,16,32 and 64mg/l) and ciprofloxacin (4,16 and 64mg/l). Ten aliquots of Brain Heart Infusion broth each was inoculated with each isolate subcultured on BHIA which had been incubated at 37°C previously. The inoculated aliquots were incubated at 37°C. One ml of a 10^2 dilution of a culture of each isolate from the overnight incubation was pipetted into a series of tubes containing 9ml of BHI broth each into which known concentration of antibiotic(s) had been incorporated. The final 10-ml volumes contain approximately 10^5 to 10^7 organisms per millilitre together with antibiotic alone or combined with another antibiotic. One tube of each isolate without antibiotic was used as control. Tubes were incubated at 37°C during the period of experiment with respect to the removal of 0.1ml each and serial dilutions. Tests were carried out in triplicate. The colony counts were performed at time: 0, 4, 8 and 24 hours by removing 0.1ml each from the broth and serially diluting approximately in saline to produce 10 fold serial dilutions (e.g 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} in order to eliminate potential carryover effect) and put one drop (approx.20µl) of each diluted saline in each corner of triangle for the marked bottom of each BHIA plate (i.e a total of three drops per triangle for each 10 fold dilution) and allowed to absorb and dry without streaking. The plates were incubated at 37°C for 24-48 hours before colonies were counted. The activity of antimicrobial alone and in combination was determined by plotting $\log_{10}$ colony counts (cfu/ml) against time.

Synergy and antagonism were defined as a greater than 2 $\log_{10}$ cfu/ml decrease or increase respectively from the original inoculum at time zero.

2.2.6 Selection of Gentamicin-resistant strains

Clinical strains were selected for further study with respect to aminoglycoside modifying enzymes (AMEs) on the basis of their MICs to gentamicin as determined by the agar dilution method in section 2.2.3. In this case, if the MIC of gentamicin exceeded 8μg/ml.

2.2.7 Identification of Aminoglycoside Modifying Enzymes by PCR

The presence or absence of the genes that encode *ACC(6')+APH(2")* for HLR to gentamicin in the clinical isolates was confirmed by amplifying specific region(s) using multiplex PCR technique. The primers used in the amplification are indicated in Table 2.4. The strain NCTC 29212 was used as the control organism. Briefly, crude DNA template for the PCR reaction was made for each isolate by boiling 0.8ml bacterial cells in 0.2ml sterile deionized water (1.0ml) obtained from the overnight culture for 10 minutes in boiling water, centrifuged for 5 minutes to remove the debris and us 20μl of supernatant used as a template in the PCR reactions. PCR was performed in 100μlchilled reaction mixture containing 2.5μl of 10x PCR buffer, 1.0mM $MgCl_2$ for *aac(6')+aph(2")*, 0.2mM deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 0.5uM each primer and 2.5U of *Taq* DNA polymerase (Advanced Biotechnologies, Dorking, Surrey). PCR was performed in a Techne Cyclogene Thermal Cycler (Cambridge) with the following conditions:-10 min at 95°C, 30s at 94°C, and 30s at 58°C and 30s at 72°Cfor 30 cycles: 10 min at 72°C and then maintained

at 4°C until analysis PCR products were electrophoresed on horizontal slab gels in the Bio-Rad subcell® GT agarose gel-electrophoresis method. The samples were first mixed with the loading buffer containing mixture of 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, and 30% w/v sucrose to the ratio of 5:1 before loading onto the gel including an appropriate molecular weight marker. Electrophoresis was run at a constant voltage of 100V until the loading buffer fronts had moved two-third down the gel. Gels were stained following electrophoresis with the solution containing concentration of 0.5μg/l ethidium bromide and visualized on a UV transilluminator. Photographs were taken using a Polaroid camera fitted with an orange filtrer.

Table 2.4 Primers for detection of aminoglycoside resistance genes

Resistance gene	Size (bp)	Position	Primer Sequence (5' – 3')
aac(6')-le-aph(2")-la	369	N/A	CAGGAATTTATCGAAAATGGTAGAAAAG
		N/A	CACAATCGACTAAAGAGTACCAATC
aph(2")-lb	867	N/A	CTTGGACGCTGAGATATATGAGCAC
		N/A	GTTTGTAGCAATTCAGAAACACCCTT
aph(2")-lc	444	N/A	CCACAATGATAATGACTCAGTTCCC
		N/A	CCACAGCTTCCGATAGCAAGAG
aph(2")-ld	641	N/A	GTGGTTTTTACAGGAATGCCATC
		N/A	CCCTCTTCATACCAATCCATATAACC

N/A= Not available

2.2.8 Screening of *E.faecalis* for a Tn*5281*-like Transposon by long-PCR Method

The chromosomal DNA was used as template to amplify a 3.5 kb fragment of *Tn*5281 by the Expand Long Temple PCR System (ABgene, Epsom, Surrey, UK) according to manufacturer's instructions. Because Tn*5281* has a gene *aac(6')+aph(2")* flanked by IS*256* sequence in inverse orientation, therefore, any PCR primer used to act as a 'forward primer' described by Dyke *et al,* 1992, would also anneal to the inverted IS256 sequence and subsequently act as a 'reverse primer'. Therefore, the L-PCR method utilized only a single IS*256* primer (5-' CAGAACAGCTGGATCCTATGG-3 restricted with *Bam*HI or 5' GTCGACTTTTAGCCTCACGCG-3' restricted with *Sal*1) for the amplification of a 468-bp sequence from within ORF390 of IS*256*. Each PCR reaction had contained 350 μM of each dNTP, 300nM of IS*256* forward primer, 5 μl of x10 reaction buffer in 1.75 mM magnesium chloride concentration, 250ng of chromosome DNA and 2.5 units of reaction enzyme (*Taq* and *Pwo* DNA polymerase). The final volume was made up to 50 μl with sterile distilled water. The amplification was performed with one cycle of 94°C for 2 min, 10 cycle of 92°Cfor 10s, 57°C for 30s and 68°Cfor 45s, 30 cycles of 92°C for 10s , 57°C for 30s and 68°C for 8 min (with the elongation time increased by 20s per cycle) and a final elongation cycle of 68°C for 7 min.

2.2.9 Detection of IS*256* Elements By L-PCR

To establish if IS*256* elements were present in the clinical isolates, L-PCR amplification of a 468 bp fragment specific to IS*256* was performed as described by Dyke *et a*l (1992) indicated in section 2.2.8.

2.2.10 Bacterial genomic DNA extraction

High quality bacterial genomic DNA was extracted with guanidium thiocyanate as described by Pitcher *et al* (1989). A single colony from each plate grown overnight was inoculated into each 10ml BHI broth and grown overnight on shaker at 37°C. The broth cultures were then harvested by centrifugation and washed in TE buffer before resuspending in 100µl TE supplemented with 50mg/ml lysozyme. The cell pellet was incubated at 37°C for 30 minutes and then lysed with addition of 0.5ml GES reagent (5M guanidium thiocyanate, 100mM EDTA and 0.5% v/v sarkosyl). Lysates were cooled on ice before adding 0.25ml of 7.5M ammonium acetate kept on ice for 10 minutes. DNA was precipated by addition of 0.54 volumes of 2-propanol kept at -20°C and collected by centrifugation. DNA pellets were washed in 70% ethanol prior to redissolving in 100ul of sterile deionized water.

2.2.11 Examination of DNA Preparation By Agarose Gel Electrophoresis

DNA preparations were examined based on their purity, quantity and size by conventional agarose-gel electrophoresis. Gel electrophoresis was performed in TAE (10x TAE pH 7.6: 40 mM Tris-acetate, 1mM EDTA) buffer. The gels were made by

melting 1% agarose in 1x TAE in a microwave oven. Gels were cast in an appropriate casting tray and after solidifying, placed in a gel electrophoresis tank containing 1x TAE buffer. Six µl of each PCR sample containing 1ul of tracking dye(30%glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol) were loaded in a well at one end of the tray along with 100bp DNA ladders (GibcoBRL, Montgomery, Maryland) as molecular weight reference. Electrophoresis at 100V was then performed for 20-30 minutes. The samples were examined by UV visualization on UV transillumination (UV Products, Cambridge). A good purity DNA showed only one band and their quality and size were estimated by comparing with the standard DNA marker(s) on the same gel.

2.2.12 Pulsed-Field Gel Electrophoresis

In order to establish relatedness among the clinical isolates, Pulsed-field gel electrophoresis (PFGE) was used to analyse the difference or non-difference among the isolates. Harvesting of cells was done by vortexing for 5 minutes the overnight BHI broth culture (2ml) each inoculated with a single colony the previous day and standardized to McFarland's 4 opacity. 400µl were transferred each to 1.5ml Eppendorf tube and spun down on microcentrifuge for 2 minutes at maximum speed (15,000 rpm), discarded the supernatant and added 800µl each of TE buffer [10 mMTris-HCL (pH 7.5)], 0.1 mM EDTA] vortexed properly and spun for 2 minutes. The supernatant was discarded and 200ul each of TE buffer and 20µl each of achromopeptidase was added and vortexed thoroughly. The added 220µl seaplaque agarose (Bio-Rad Laboratories, UK), vortexed briskly and loaded each to block mould. Plugs were allowed to set in

fridge for 15 minutes before dislodging the plugs from each mould into each bijou tube containing pre-warmed lysis buffer in 50°C water bath and left for one hour. Washing plugs was done by first discarding lysis buffer and adding 2 ml of pre-warmed TE buffer while keeping the plugs at 50°C water for 15 minutes. The TE buffer was changed with fresh 2ml TE buffer and the plugs left at room temperature for 15 minutes. Fresh 2ml TE buffer was added and left at room temperature for another 15 minutes before storing in TE buffer (2ml) at 4°C indefinitely. DNA digestion was done by first labelling 0.5ml Eppendorf tubes accordingly. Agarose plugs were cut approximately 5mm x 3mm in size each and placed into each tube then 100µl of a 1/10dilution of *Sma*1 buffer A was dispensed into each tube before adding 2µl *Sma*1 restriction endonuclease (Promega Southampton, UK) into each tube. Tubes were incubated at 30°C overnight. Prepared 1.2% PFGE certified agarose in x 0.5 TBE buffer (ie 1.2g agarose in 100ml of half strength TBE) before dissolving in the microwave oven and pouring into tray. A comb was inserted and the gel allowed to set. Lambda molecular weight markers were added and pre-warmed at 52°Cfor 8 minutes. Each well was loaded with each restricted product including marker. All wells were sealed with the saved agarose and the gel kept for 10 minutes in the fridge to solidify before putting into PFGE tank and secured. TBE (1900ml) was poured into the tank and covered the tank. Electrophoresis was performed in a contour-clamped homogeneous electric fields device (CHEF-DR11; Bio-Rad Laboratories LTD, UK). The gel was set to run at initial pulse of 5s and final pulse of 40s for 24 hours. Pump was set at 65, 12°C, voltage at 200V. At the end of run, gels were stained with solution containing concentration of ethidium bromide (0.5ug/ml) for 15 minutes and destained in distilled water (2x15 minutes) before being visualized under UV illumination and took photographs or scanned gel image in computer.

2.2.13 Interpretation of PFGE Banding Patterns

2.2.13.1 PFGE banding patterns by visual comparison

PFGE banding patterns were compared visually based on the interpretation by the criteria of Tenover *et al* (1995). Thus, isolates are deemed indistinguishable if they have identical PFGE banding patterns. Isolates with differing banding patterns by up to three bands are considered to be closely related and four to six banding differences are assumed to be 'possibly related'. Isolates with seven or more bands bands difference are considered to be unrelated.

2.2.13.11 Comparing PFGE Banding Patterns with aid of Computer

Banding patterns were compared with the Bionumeric Software version 3.0 Diversity Database software based on introduction of gel images into the Diversity Database from the original Polaroid photographs. Analysis was then performed based on guidelines from themanufacturer resulting into phylogenic trees.

2.2.14 Random Amplification Polymorphic DNA (RAPD) PCR

RAPD also known as arbitrarily primed PCR was to show similarities or differences between *E.faecalis* and *E.faecium* with respect to bandings. Extraction of DNA was done based on the method in section 2.2.10. A single primer sequence used was 5'-GAGGTGGCGGTTCT-3'. PCR reaction mixture consisted of 2µl of sample, 2.5µl primer, 2.5µl *Taq*, 2.5µl of 10x PCR buffer, 1µ $MgCl_2$, 10µl each of dNTPs , 20µl

tris-HCl at pH8.3 and 32µl MQ H_2O. Amplification was done by one cycle at 90°C for 10 minutes, 94°C for 1min, 45°C for 20s, 72°C for 54s in 40cycles and then followed by one cycle at 72°C for 2 minutes. The PCR products were run on electrophoresis before staining with ethidium bromide solution (0.5µg/ml) and visualized by UV. Photographs were taken using a Polaroid camera and the bandings were compared with those obtained from PFGE method.

2.2.15 Identification of *gyr*A and *par*C resistance among the isolates by PCR

The objective was to amplify the QRDR segment of *gyr*A and *par*C genes of the isolates in order to sequence the QRDR. The PCR was performed using the primers indicated in Table 2.5. A DNA fragment of 241 bp from *gyr*A gene corresponding to quinolone resistance determining region was amplified with primers indicated in Table 2.5 equivalent to nucleotide positions 150 to 172 and 368 to 390 of *E.coli gyr*A gene respectively. A 191-bp *par*C fragment was amplified with the primers also indicated in Table 2.5 with position 10 to 229 and 181 to 200 of the *E.faecalis par*C respectively. One-step PCR was performed for each gene in a 50µl reaction mixture containing 1.25 U of Taq DNA polymerase, 200µm each deoxynucleoside triphosphate, 45pmol of each primer, 1.5mM MgCl2 and 10x PCR buffer (Sigma). Each reaction was run for 30 cycles with the following temperature profile : denaturation at 94°C for 1 min, annealing at 55°C for *gyr*A and 48.7°C for *par*C for I min; and then extention at 72°Cfor 1 min. ATCC 29212 was used as a reference. The amplified fragments were analysed in ABI 377 prism automated sequencer.

Table 2.5 Primers used for detection of gyrA and parC genes by PCR of *E.faecalis*

Amplified gene	Primer	Sequence 5' – 3'	size of amplicon	Position
gyrA	A_1	CGGGATGAACGAATTGGGTGTGA	241bp	150-172
	A_2	AATTTTACTCATACGTGCTTCGG		368-390
parC	C_1	AATGAATAAAGATGGCAATA	191bp	10- 29
	C_2	CGCCATCCATACTTCCGTTG		181-200

1=Forward Primer 2=Reverse Primer

CHAPTER 3 : Results

3.1 Characterization of clinical isolates of Enterococci

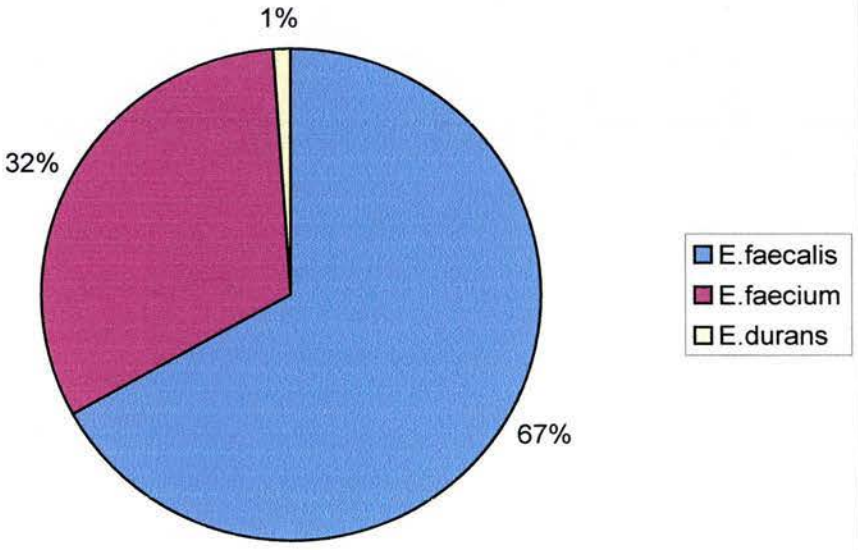
Enterococci, especially *E.faecalis* and *E.faecium*, are the second leading cause of nosocomial infections (Schaberg *et al.*, 1991). The identification of an *Enterococcus* isolate to species level is clinically crucial for the proper patient management and for the epidemiology purposes.

3.1.1 Speciation of the clinical isolates by API 20 Strept system

The API 20 Strept system is one of a series of miniaturized classical biochemical tests from commercial sources widely used by clinical laboratories across the world for the identification of many groups of organisms (bio-Merieux). The system is simple and gives consistent results although it may not recognize some of the more recently described enterococcal species because of the phenotypic similarities among the strains and has failed to identify *E.gallinarum* and *E.casseliflavus* (Hamilton-Miller & Shah,1999). Therefore, further testing is necessary to confirm the species identification. 81 clinical isolates (ie 55 *E.faecalis* and 26 *E.faecium*) were identified by the API 20 Strept system based on profile index in the computer-based identification services and were found to be *E.faecalis* (67%), *E.faecium* (32%), and *E.durans* (1%) (Fig 3.1) and (See also Appendix A). The isolates were previously identified with the aid of API 20 S by the clinical laboratories as either *E.faecalis* or *E.faecium* at the time of their isolation from the clinical specimen. Further identification of isolates was confirmed by PCR (section 3.1.2). Many pathogens including *E.faecalis* produce haemolysis capable of destroying erythrocytes , for example on horse blood agar. The significance of haemolysis in pathogenicity is not

yet understood. However, such haemolytic patterns could be used as a cultural characteristic on the blood agar which gives one of the clues of the identification processes of the isolates in the clinical laboratory settings which frequently receive and process such samples. The clear haemolytic zone around the colony in the blood agar indicates a complete haemolysis of red blood cells (erythrocyes) called "β-haemolysis". The greenish-grey zone around colonies on the blood agar attributed to partial lysis of RBCs is called "α-haemolysis". All *E.faecium* (26) showed α- haemolytic on horse blood agar and *E.faecalis* (55) showed mixture of α (21) and β (34) haemolysis on horse blood agar (appendix A).

Fig 3.1 The Percentage Identification of Isolates by species



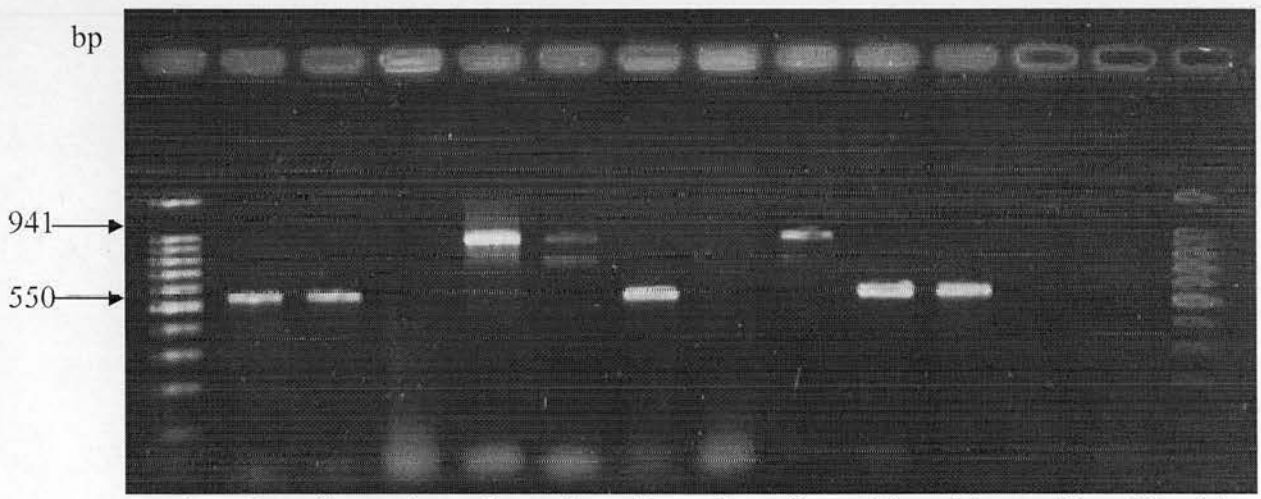
3.1.2 Identification of two enterococcal species by PCR method

The use of PCR for identification of enterococcal species was based on specific detection of genes that encoded D-Alanine:D-Alanine (D-ala:D-ala) ligases. The PCR assay relies on specific amplification of fragments intragenic to $ddl_{E.faecalis}$ and $ddl_{E.faecium}$. Dutka-Malen *et al.* (1995), showed that the $ddl_{E.faecium}$ and $ddl_{E.faecalis}$ genes are specific for *E.faecium* and *E.faecalis* and, therefore, could be used for identification of the two species. The two pairs of primers (Table 2.3), each intended to prime amplification of a fragment internal to a gene, were selected based on nonconserved regions and each amplification product was assigned to a gene on the bases of its size that can be readily identified by migration electrophoretic gel analysis (Dutka-Malen *et al*,1995). PCR was performed with DNA from every isolate as template against the primers (Table 2.3) . One band was generated as PCR product with size of 550 bp resulting from amplification portion of the $ddl_{E.faecium}$ observed with each DNA template from *E.faecium* isolates (Fig 3.2) . The quality of gels with respect to one *E.durans* isolate-17B/377 and *E.faecium* isolates 17B/235, 17B/721, 17B/849, 18B/33, NCTC12202*, 18B/89*, 18B/149, 18B/234, 18B/254, 18B/294 and 18B/298 appear to be very good but there was no banding from isolates with *(asterick) mark due to non reactivity between extracted isolates DNA with primers in the PCR mixture or otherwise. Similarly, one band of 941 bp was generated from amplification of $ddl_{E.faecalis}$ when each DNA template from *E.faecalis* isolates was used (Fig 3.2). The quality of gels for *E.faecalis* isolates 17B/407*, 17B/452, 17B/686, 17B/761, 17B/819, NCTC29212, 18B/309*, 18B/314, 18B/329*, 18B/337*, 18B/358* and 18B/376* appear to be ranging from good to very good and again each isolate with

astrerick mark did not produce banding due to inactivity in the PCR mixture. However, there was no difference between *E.faecium* and *E.durans* by PCR method (lane 2-*E.faecium* and lane 3-*E.durans* Fig 3.2). Both had shown a band size of 550bp. This contrasted to identification of *E.faecium* and *E.durans* by API 20 Strept system (Section 3.1.1)and reveals a flaw in the PCR method . An attempt to sequence both species from the PCR product to differentiate the two species did not produce a clear result. This investigation could not show that this method was sufficiently reliable for the differentiation of *E.faecium* and *E.durans*, but it was useful to distinguish *E.faecium* from *E.faecalis*. This distinction of *E.faecium* from *E.durans* has been from phenotypic methods only. Lane 4, 8,12 &13 in Fig 3.2 (a) and lane 8, 10-13 of (b) had no banding. NCTC29212 & NCTC12202 were used instead of NCTC 12697 and NCTC 7171 as controls because all isolates were found to be gentamicin resistance.

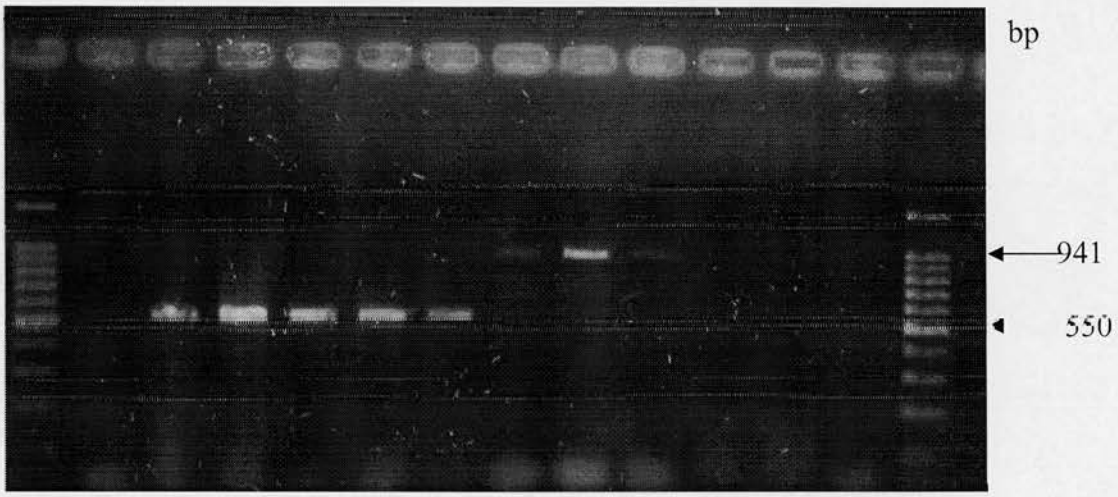
Fig 3.2 (a) & (b) Analysis of agarose gel electrophoresis of amplified *ddl* $_{E.faecium}$ and *ddl*$_{E.faecalis}$ PCR Products

(a)



Lane1-λ ladder; 2-RIE-17/235; 3-RIE-17/377; 4-RIE-17/407;5-RIE-17/452; 6-RIE-17/686; 7- RIE-17/721; 8-RIE-17/761;9-RIE-17/819; 10-RIE-17/849; 11-RIE-18/33; 12-NCTC12202;13-NCTC29212; 14-λ ladder

(b)



Lane 1-λ ladder; 2- RIE-18/89; 3-RIE-18/149;4-RIE-18/234; 5-RIE-18/254; 6-RIE-18/294; 7-RIE-18/298; 8-RIE-18/309; 9-RIE-18/314; 10-RIE-18/329; 11-RIE-18/337;12-RIE-18/358; 13-RIE-18/376; 14-λ ladder

3. 2 Analysis of the Isolates by PFGE Method

Several techniques have been used in medical microbiology for acquiring information on the spread of pathogenic bacteria within the hospital environment and outside in the community (Antonishyn *et al*, 2000). The PFGE technique used in this study was to analyze the degree of clonality with the gentamicin-resistant isolates (52 *E.faecalis* and 21 *E.faecium*) obtained from RIE. The PFGE technique is considered to be most reliable due to its discriminatory power, sensitivity and reproducibility (Gordillo *et al*,1993; Murray *et al*, 1990; Chiew & Hall,1998) The interpretation was done based on the criteria described by Tenover *et al,*(1995). The criteria are only used in the hospital laboratories when examining relatively small sets of isolates (ie not more than 30) which are related to putative outbreak of the disease. The interpretation

criteria was used to show the number of fragment differences that would be expected within the PFGE patterns showing a defined number of genetic occurrences either as point mutation resulting in the creation or loss of restriction sites and insertions or deletion of DNA. Thus, PFGE patterns that differ by two or three fragments are deemed to be closely related on the differences that occur through a single genetic event. According to Tenover *et al*, 1995, two genetic events would result into four to six fragment differences showing unrelatedness and these criteria are reliable if PFGE resolves at least ten distinct fragments. Accordingly, a point mutation that would lead to the criteria of an additional restriction site may result in a three fragment differences. This might be taken as closely related to the original pattern. However, transfer of large chromosomal elements associated with vancomycin resistance could alter the biochemical properties of the strain (McAshan *et al,*1999). The study by Morrison *et al*, 1999 showed that a single strain can differ by up to seven fragments based on the temporal , association of the isolates representing a single strain. If the criteria defined by Tenover *et al*, 1995, were widely accepted, they would not be applied to all bacterial species. Therefore, it was with the hope or anticipation that isolates would fail to be recognized as related by the Bionumeric Software version 3.0. Indeed, that was what had occurred in the visual comparison with the Bionumeric Software version 3.0 computer among the *E.faecium* isolates involved in this study. The computer software identified *E.faecium* isolates RIE-17B/849 and RIE-18B/33 with 80% similarity while RIE-18B/517 and RIE-18B/567 were identified with 70% similarity. RIE-18B/815 and RIE-19B/471 were identified with 66% similarity. Isolates RIE-18B/536 and RIE-18B/632 had only 64% similarity. RIE-17B/235 and RIE-18B149 were identified as 40% similarity. On the other hand, *E.faecalis* isolates RIE-17B/761 and RIE-17B/819 were identified by computer software with similarity of 90% while RIE-17B/686 and

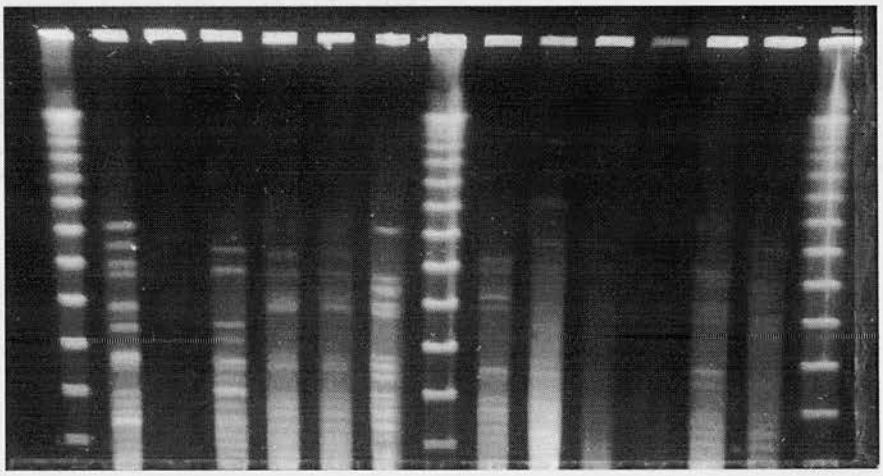
RIE-18B/329 identified with 80% similarity. RIE-18B/337 and RIE-18B/358 had 76% similarity. All the isolates identified as unrelated to any other isolates by visual comparison of PFGE patterns were confirmed by computer software as unrelated. However, the computer software calculates that the two isolates, for example, have 66% similarity, such a percentage similarity shows that the strains are not related at all although it may be that the percentage may be significant given the variety observed in the percentage similarity of identical strains.The PFGE technique is considered to be most reliable due to its discriminatory power, sensitivity and reproducibility (Gordillo *et al,*1993; Murray *et al*, 1990; Chiew & Hall,1998). Twelve of the 52 *E.faecalis*, 21 *E.faecium* and one *E.durans* isolates were analysized by PFGE method to explore relatedness among the strains.

3.2.*1* Visual analysis of PFGE Patterns

Initially banding patterns were compared by eye and interpreted using criteria described by Tenover *et al*, 1995. These criteria indicate that isolates are deemed to be indistinguishable if they have identical banding patterns. Isolates with upto three bands differences are considered to be closely related and with four to six bands differences would indicate 'possibly related'. Isolates are considered unrelated if they have bands differences of seven or more. To overcome difficulties in comparing banding patterns on different gels, the different PFGE types identified were re-run on the same gel. Fig 3.3 shows the main PFGE types recognized and Table 3.1 (a) & (b) and Table 3.2 assigned PFGE types as determined in the visual comparison of restricted patterns. All isolates were obtained from the Royal Infirmary of Edinburgh (RIE). Visual comparison of the PFGE patterns for *E.faecalis* [Fig 3.3(c)] showed that all isolates were identical except RIE-18/309 lane-7. The identical isolates appeared to be the outbreak strain and were

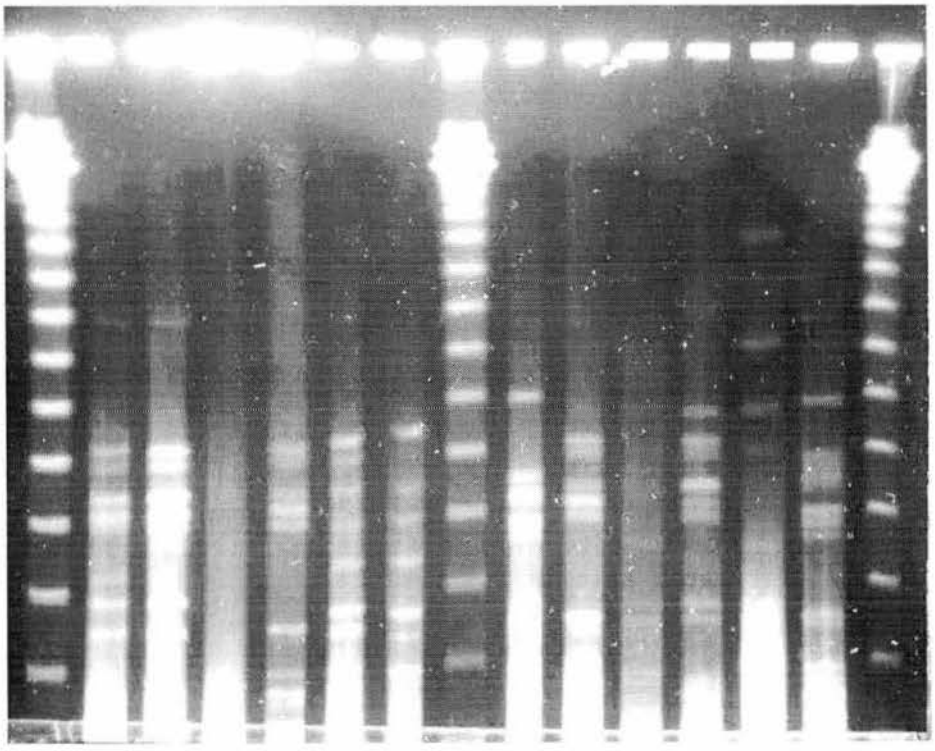
from the same ward. However, RIE-18/309 appeared to have come from a different ward. There were variations with regards to *E.faecium* isolates and they were collected from different wards within the RIE [Fig 3.3 (a)]. Isolates in Lanes 3&12 had no band. RIE-17/849, RIE-18/33 and RIE-18/234 appeared to be identical. RIE-17/235, RIE-18/149, RIE-17/721 each showed different bands from the rest of the isolates thus indicating the heterogeneity of the isolates. Fig 3.3 (b) also showed heterogeneity with respect to the bandings.

Fig 3.3 (a),(b) & (c) PFGE patterns of *Sma*1 digested chromosomal DNA of Isolates (a)



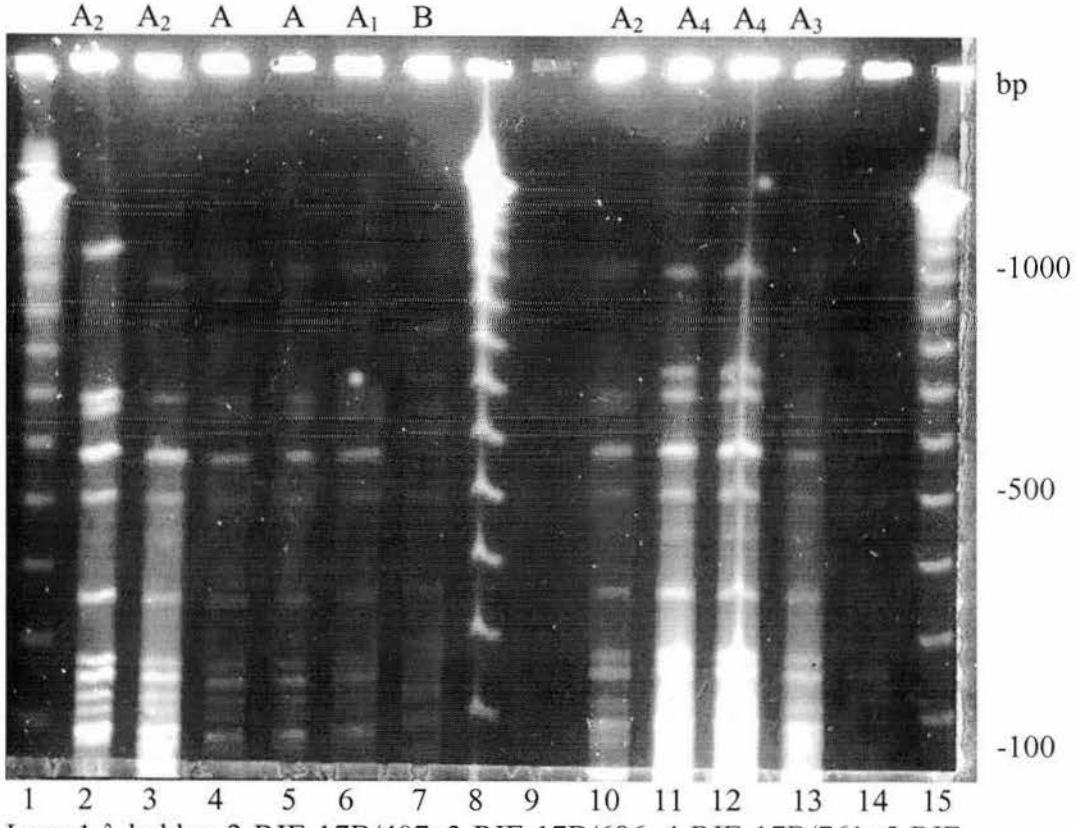
Lane 1-λ ladder; 2-RIE-17/235; 3-RIE-17/377; 4-RIE-17/721; 5-RIE-17/849;6-RIE-18/33; 7-RIE-18/149; 8-λ ladder;9-RIE-18/234; 10-RIE-18/254; 11-RIE-18/294; 12-RIE-18/298;13-RIE-18/487; 14-NCTC122202; 15-λ ladder (All *E.faecium*)

(b)



Lane 1-λ ladder; 2-RIE-18B/517; 3-RIE-18B/536; 4-RIE-18B/567; 5-RIE-18B/632; 6-RIE-18B/662; 7-RIE-18B/749; 8-RIE-λ ladder; 9-RIE-18B/813; 10-RIE-18B/815; 11-RIE-18B/960; 12-RIE-19B/391; 13-RIE-19B/471; 14-RIE-NCTC12202; 15-RIE-λ ladder (All *E.faecium*)

(c)



Lane 1-λ ladder; 2-RIE-17B/407; 3-RIE-17B/686; 4-RIE-17B/761; 5-RIE-17B/819; 6-RIE-18B/89; 7-RIE-18B/309; 8-λ ladder; 9-RIE-18B/314; 10-RIE-18B/329; 11-RIE-18B/337; 12-RIE-18B/358; 13-RIE-18B/376; 14-RIE-18B/379; 15-λ ladder (All *E.faecalis*)

Table 3.1 PFGE patterns of *E.faecium*

(a)	Lane #	Isolate #	Species	PFGE pattern
	1			λ ladder
	2	17B/235	*E.faecium*	A
	3	17B/377	*E.durans*	
	4	17B/721	*E.faecium*	B
	5	17B/849	*E.faecium*	A_1
	6	18B/33	*E.faecium*	A_1
	7	18B/149	*E.faecium*	D_2
	8			λ ladder
	9	18B/234	*E.faecium*	A_2
	10	18B/254	*E.faecium*	A_3
	11	18B/294	*E.faecium*	A_4
	12	18B/298	*E.faecium*	
	13	18B/487	*E.faecium*	A_1
	14	NCTC12202	*E.faecium*	C_3
	15			λ ladder

(b)	Lane #	Isolate #	Species	PFGE pattern
	1			λ ladder
	2	18B/517	*E.faecium*	C_2
	3	18B/536	*E.faecium*	C_2
	4	18B/567	*E.faecium*	C_2
	5	18B/632	*E.faecium*	C_2
	6	18B/662	*E.faecium*	C
	7	18B/749	*E.faecium*	C
	8			λ ladder
	9	18B/813	*E.faecium*	C_1
	10	18B/815	*E.faecium*	C_1
	11	18B/960	*E.faecium*	D_1
	12	19B/391	*E.faecium*	D
	13	19B/471	*E.faecium*	C_1
	14	NCTC 12202	*E.faecium*	C_3
	15			λ ladder

Table 3.2 PFGE pattern of *E.faecalis*

Lane #	Isolate #	Species	PFGE pattern
1			λ ladder
2	17B/407	*E.faecalis*	A_2
3	17B/686	*E.faecalis*	A_2
4	17B/761	*E.faecalis*	A
5	17B/819	*E.faecalis*	A
6	18B/89	*E.faecalis*	A_1
7	18B/309	*E.faecalis*	B
8			λ ladder
9	18B/314	*E.faecalis*	
10	18B/329	*E.faecalis*	A_2
11	18B/337	*E.faecalis*	A_4
12	18B/358	*E.faecalis*	A_4
13	18B/376	*E.faecalis*	A_3
14	18B/379	*E.faecalis*	
15			λ ladder

3.2.2 Computer-Aided Analysis of PFGE Patterns

The Bionumeric software version 3.0 (Applied Maths, Ghent, Belgium) was used in the PFGE computer-aided analysis. The software possesses the algorithms necessary for gel analysis. The comparison and preparation of a dendogram and cluster analysis of digested profiles of isolates were based on the Dice coefficient using the hierarchic un-weighted pair arithematic average algorithm with 1.6% tolerance. Fragments smaller than 48.5kb in length were not used in analysis. The criteria defined by Tenover *et al*, (1995) are intended for use in the investigation of relatively small sets of isolates (approx $\leq$30) related to the putative outbreaks of infection (Tenover *et al,* 1995). Therefore, they are not suitable for the study of all the isolates. The PFGE interpretation will be given in the discussion (Chapter 7). The gel images introduced into Bionumeric software version 3.0 were taken from the original Polaroid photographs. Unfortunately, gels were not initially run with a view to making the computer-aided comparison. However, in a good setting, the gels should be run using two DNA ladders flanking the samples and another DNA ladder situated in the middle of the gel. The cluster analysis of data was performed on two gel photographs indicated in Fig3.4 (a) and (b). It was with the hope that isolates deemed to be related by visual comparison of PFGE patterns would fail to be recognized as related by the computer software. Indeed, that was what had occurred in the results indicated in Fig 3.4 (b). Instead of having one outbreak strain as indicated in the visual comparison, the phylogenetic tree obtained through Bionumeric software version 3.0 suggested several small clusters of related isolates . Similarity judged by eye as identical range

approximately 58% to 95%. However, when analysed by Software, similarity appears to be different [Fig3.4 (b)]. Bionumeric software identified RIE-17B/761 and RIE-17B/819 [Fig3.4 (a) *E.faecalis*] as identical (90% similarity). Isolates RIE-17B/686 and RIE-18B/329 were identified as identical (80% similarity). RIE-18B/337 and RIE-18B/358 had only 76% similarity. In Fig 3.4 (b) for *E.faecium*, Bionumeric software identified RIE-17B/849 and RIE-18B/33 as identical (80% similarity). RIE-18B/517 and RIE18B/567 showed similarity of 70% and closely related isolates RIE-18B/815 and RIE-19B/471 showed 66% similarity . Isolates RIE-18B/536 and RIE-18B/632 had only 64% similarity. Isolates 17B/235 and 18B/33 showed 40% similarity All the isolates identified as unrelated to any other isolates by visual comparison of PFGE patterns were confirmed by Bionumeric software version 3.0 analysis as unrelated. When the calculation by Bionumeric software analysis shows that the two isolates have 66% similarity, such percentage similarity would mean that strains are not related although it might be that the percentage may be significant given the variation observed in the percentage similarity of identical strains.

Fig 3.4 (a) Cluster analysis of DNA Banding Patterns of *E.faecalis*

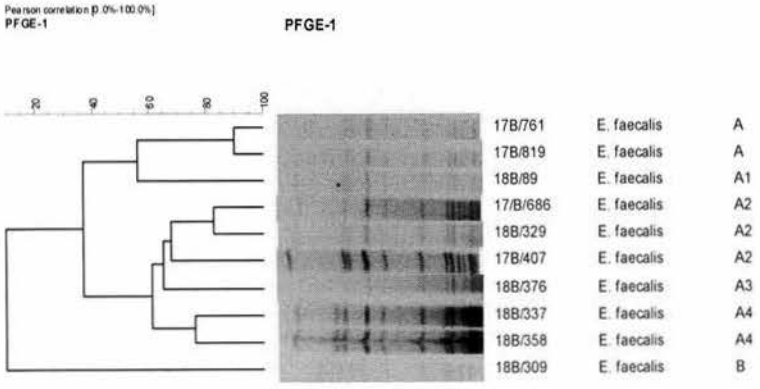
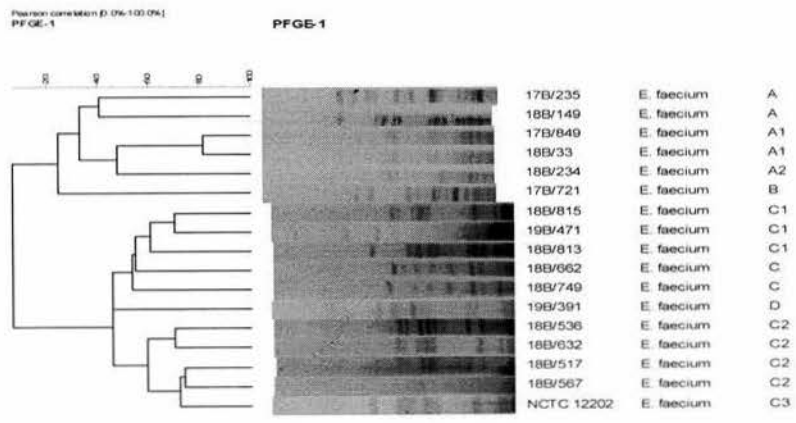


Fig 3.4 (b) Cluster analysis of DNA Banding Patterns of *E.faecium*



* Isolate 18B/149 had visual banding pattern of D_2 instead of A

Table 3.3 (a) & (b). PFGE patterns of Computer-Aided % Similarity Analysis

(a) *E.faecalis*

Isolate	Percentage
17B/761 + 17B/819	90%
17B/686 + 18B/329	80%
18B/337 + 18B/358	76%

(b) *E.faecium*

Isolate	Percentage
17B/235 + 18B/149	40%
17B/849 + 18B/33	80%
18B/815 + 19B/471	66%
18B/536 + 18B/632	64%
18B/517 + 18B/567	70%

3.3 The Comparison of RAPD method with PFGE Technique

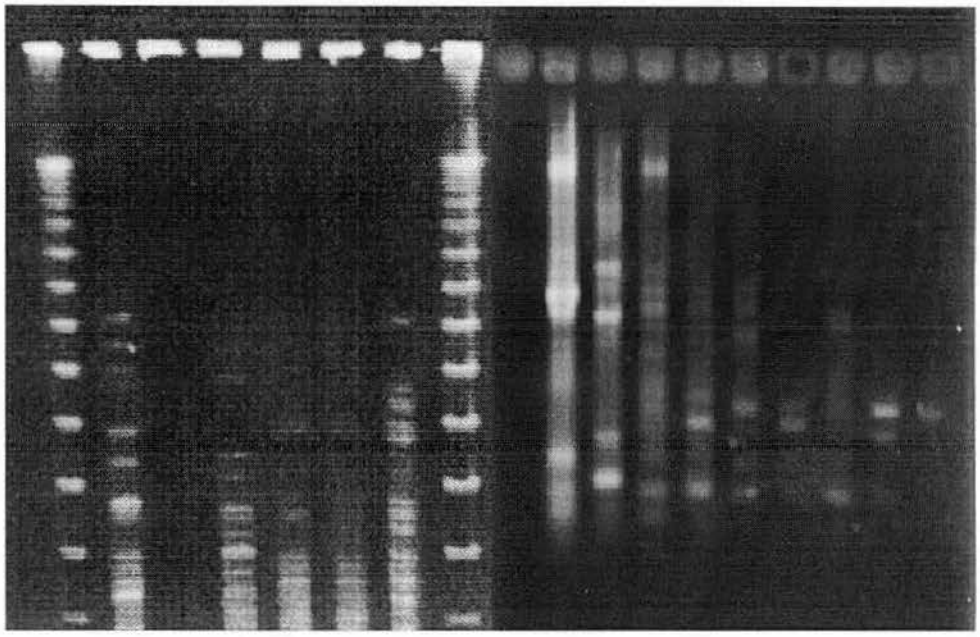
Both RADP and PFGE techniques have been used to investigate the epidemiology of enterococcal infections (Chiew and Hall, 1998). PFGE technique is based on the digestion of chromosomal DNA with a restriction endonuclease that cleaves infrequently and produces only a few high-molecular-weight fragments that can be separated under special conditions of electrophoresis. On the other hand, AP-PCR (RAPD) is based on non-specific random amplifications by PCR of the bacterial chromosome using a short primer under low-stringency conditions. The RAPD method was compared with the PFGE method with respect to equal Discrimination (Bingen *et al*,1996). The banding system was used as a way of showing the discriminating systems between the two

techniques. Few isolates were selected for the comparison of the two techniques (Fig 3.5). The comparison was done using PFGE (a) for the strains RIE-17/235, RIE-17/377, RIE-17/407, RIE-17/452, RIE-17/686 and RIE-17/721 and with the RAPD (b) for the strains RIE-17/849, RIE-18/33, RIE-18/89, RIE-18/149, RIE-18/234, RIE-18/254, RIE-18294, RIE-18/294,RIE-18/309 and RIE-18/314. Banding between lane 2(a)-RIE-17/235 (*E.faecium*) and lane 2(b)-RIE-18/33 (*E.faecium*) showed that strain RIE-17/235 had the same bandings (15) with the strain18/33 (15 bandings). There was no banding observed between strain RIE- 17/377 (*E.durans*) in lane 3 (a) and strain 17/849 (*E.faecium*) in lane 1(b). The strain RIE-17/407 (*E.faecalis*) in lane 4(a) had 12 bandings as compared to strain RIE-18/89 (*E.faecalis*) with 15 bandings. The strain RIE-17/452 (*E.faecium*) in lane 5(a) had 13 bands compared to strain RIE-18/149 (*E.faecium*) in lane 4(b) which had 14 bandings. The strain RIE-17/686 (*E.faecalis*) in lane 6(a) had 12 bands as compared with strain RIE-18/234 (*E.faecium*) in lane 5(b) which had 10 bands. The strain RIE-17/721 (*E.faecium*) in lane 7(a) had 17 bandings as compared to strain RIE- 18/254(*E.faecium*) in lane 6(b) with 14 bandings. RIE – 17/294 (*E.faecium*) lane 7(b) had 7 bandings. Lane 8(b) RIE-18/298 *(E.faecium*) had 8 bands while RIE-18/309 (*E.faecalis*) lane 9(b) had six bands. RIE- 18/314 (*E.faecalis*) lane 10(b) had only three bandings. The differences between the bandings among the compared strains appeared not to be large. In all, PFGE generated more bandings than RAPD observed between the two techniques. One potential criticism of RAPD (AP-PCR) is that, the bands generated are the result of arbitrary and potentially mismatching priming events (Collier *et al*, 1996). The differences in banding patterns may occur as a consequence of variations in experimental conditions beyond the control of the operator (Collier *et al,* 1996). These artificial variations would compromise the values of interference made regarding the extent of genetic variation

among the strains based on similarity or lack of AP-PCR patterns. However, in order to reduce operator induced variability such as found in RAPD, a technique such as Multilocus sequence typing (MLST) could, instead, be used since it has a low level of nucleotide variation. The procedure is essentially an updated version of multilocus enzyme electrophoresis (MLEE) which indexes variation within multiple core metabolic (housekeeping) genes on the basis of differing electrophoretic mobilities of the gene products (Feil *et al*, 2004). Whistle, the drawback of MLEE and other gel-based methods, for example pulsed-field gel electrophoresis, is that it is often difficult to compare results between laboratories. Whereas such a problem does not arise with MLST because of indexing directly housekeeping genes by nucleotide sequencing the internal gene fragments. Although RAPD analysis is rapid and cost effective, its reproducibility is subject of debate. The factors affecting reproducibility are extraction methods employed for DNA (Gomez-lus *et al*, 1993), concentration of template DNA (Davin-Repli *et al,* 1995) and selection of primer, polymerase and cycling conditions (Kerr, 1994). On the other hand, PFGE has been described as being nearly the optimal typing method (Maslow *et al*, 1993). It provides a highly reproducible restriction profile that shows distinct well-resolved fragments of the entire bacterial chromosome in a single gel. Several studies showed that PFGE in comparison to a variety of phenotypic and other molecular epidemiological typing methods is more superior (Goering *and* Duensing, 1990; Saulnier *et al*, 1993). PFGE has also been referred to as the gold standard for epidemiological analysis of nosocomial infection (Goering, 1993). The disadvantages of PFGE technique include the time-cosuming aspects, technical difficulty demanding and the need for expensive equipment

Fig 3.5 Comparison of Bandings between (a) PFGE and (b) RAPD of Clinical Isolates

(a) (b)



1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 9 10

Lane-1- ladder; 2-RIE-17/235; 3-RIE-17/377; 4-RIE-17/407; 5-RIE-17/452; 6-RIE-17/686; 7-RIE-17/721; 8- ladder.

Lane-1-RIE-17/849; 2-RIE-18/33; 3-RIE-18/89; 4-RIE-18/149; 5-RIE-18/234; 6-RIE-18/254; 7-RIE-18/294; 8-RIE-18/298; 9-RIE-18/309; 10-RIE-18/314.

CHAPTER 4 RESULTS: Susceptibility Testing

4.1 The *in vitro* Susceptibility Tests

In vitro susceptibility testing of bacteria remains one of the most important functions of the clinical microbiology laboratories. The central focus is on the interaction between the "bacteria" and the "drug" in the *in vitro* situation. The results of such tests have enormous hospital-wide implications, influencing decisions on the treatment of individual patients, development of antibiotic formularies and application of infection control policies (Johnson,1993).

4.1.1 Agar dilution method

The agar dilution procedure has been successfully adapted for routine use in large laboratories. This involves testing various concentrations of antibiotic based on standardized suspension of bacteria inoculated onto a series of agar plates each containing a different concentration of antibiotic encompassing the therapeutic range of the drug. The plates are then incubated at 37°C for 18-24hours. The results are interpretated in MIC showing the lowest concentration of drug preventing visible growth after incubation. All the clinical isolates (i.e 55 *E.faecalis* and 26 *E.faecium*) were tested against the antibiotics indicated in section 2.1.4 . The results were shown at MIC_{50} and MIC_{90} [Table 4.1 (a) and (b); and a combined graph Fig 4.1]. The MIC_{50} indicated values of strains at the median of the series but on its own, is not significant with respect to sensitivity or resistance. On the other hand, the more significant values were at MIC_{90} which shows the concentration required to inhibit 90% of the bacteria.

This is a much more sensitive indicator as to whether resistance was beginning to emerge in a bacterial population.

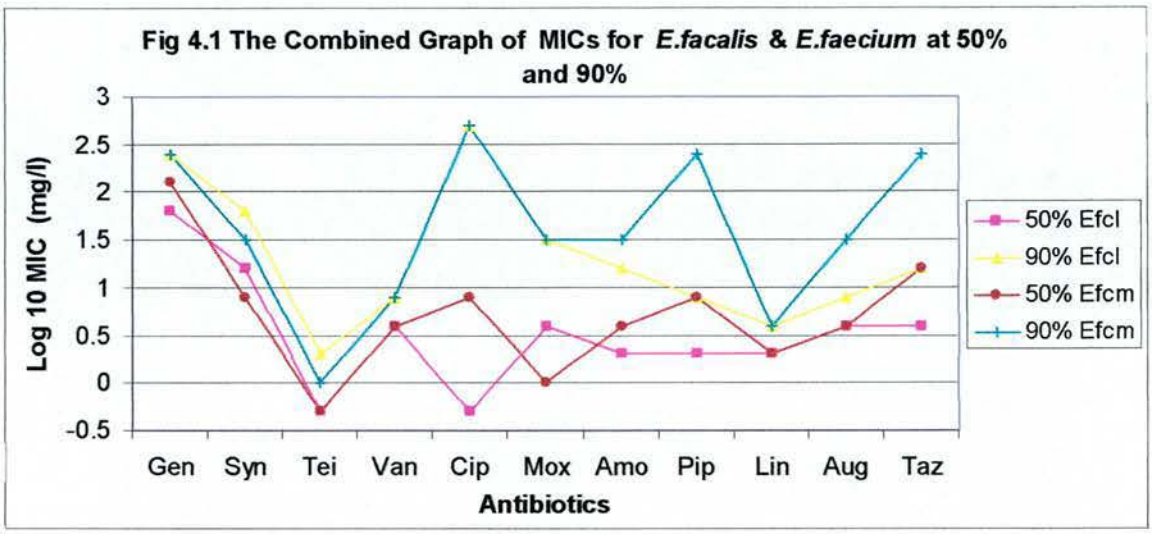
Table 4.1 (a) & (b) Antibiotics at MIC_{50} and MIC_{90} for *E.faecalis* and *E.faecium*

(a) *Enterococcus faecalis*

Antibiotic	MIC Range	MIC_{50}	MIC_{90}
	(mg/l)		
Gentamicin	32->256	64	256
Synercid	4-128	16	64
Teicoplanin	<0.25-16	0.5	2
Vancomycin	4-8	4	8
Ciprofloxacin	<0.25->256	0.5	>256
Moxifloxacin	<0.25-64	4	32
Amoxicillin	0.5-32	2	16
Piperacillin	1-16	2	8
Linezolid	2-4	2	4
Augmentin	0.5-32	4	8
Tazocin	1-32	4	16

(b) *Enterococcus faecium*

Antibiotics	MIC Range	MIC_{50}	MIC_{90}
	(mg/l)		
Gentamicin	128->256	128	256
Synercid	4-64	8	32
Teicoplanin	<0.25-2	0.5	1
Vancomycin	4-8	4	8
Ciprofloxacin	0.5->256	8	>256
Moxifloxacin	<0.25-64	1	32
Amoxicillin	0.5-64	4	32
Piperacillin	1->256	8	256
Linezolid	2-4	2	4
Augmentin	0.5-64	4	32
Tazocin	4->256	16	256



Efcl = *E.faecalis*, *Efcm* = *E.faecium*

In this study, as expected, ciprofloxacin resistance, besides the gentamicin resistance , was more common (36 *E.faecalis* and 14 *E.faecium* with each MIC >256mg/l). Both *E.faecalis* and *E.faecium* were sensitive to teicoplanin in agreement with the findings by Cercenado *et al*, 2001. All the isolates were found to be sensitive to linezolid in agreement of findings by Eliopoulos *et al*, 2002. Both *E.faecalis* and *E.faecium* were found to be moderately resistant to moxifloxacin (MIC =32mg/l). *E.faecium* isolates were more resistant to piperacillin than *E.faecalis*. Both *E.faecalis* and *E.faecium* were found to be moderately sensitive to vancomycin in agreement with the findings of Cercenado *et al*,2001. Both *E.faecium* and *E.faecalis* were found to be moderately resistant to synercid (MIC=32mg/l for *E.faecium* and MIC=64mg/l for *E.faecalis*). However, *E.faecium* were found to be more resistant to tazocin than *E.faecalis* and also *E.faecium* were found to be more resistant to augmentin than *E.faecalis*. The characteristics of *E.faecium* isolates being more resistant to β-lactam drugs than

E.faecalis appear to reflect the intrinsic resistance of *E.faecium* to the drugs. In this study, isolates with MICs >256mg/l (ie 512mg/l) were regarded as HLGR and those with MICs 8mg/l to <256mg/l were regarded as Moderately resistant to gentamicin (MLGR) in line with the findings of other researchers (Traub *et al*, 1993; Bantar *et al*,1993; Ferrara *et al*,1996; Kao *et al,* 2000). The high-level gentamicin resistance (MIC ≥1000mg/l) was first reported in France in 1979 (Horodniceanu *et al*,1979). This resistance is often associated with the production of a bifunctional enzyme that confers resistance to aminoglycosides except streptomycin (Ferretti *et al*,1986). But in streptomycin, adenyltransferase enzyme which causes the resistance to isolates is produced by HLGR strains in addition to the bifunctional enzyme. Fifty-five *E.faecalis* were found to be resistant to gentamicin (MIC_{50}=64mg/l and MIC_{90} =256mg/l; range 32- >256mg/l;Breakpoint = 4mg/l). 26 *E.faecium* were also found to be resistant to gentamicin (MIC_{50} =128mg/l and MIC_{90} = 256mg/l; range 128- >256mg/l; breakpoint =4mg/l). Both *E.faecalis* and *E.faecium* were highly sensitive to teicoplanin (MIC_{50} =<0.25mg/l and MIC_{90} =0.5mg/l; breakpoint =2mg/l for *E.faeclis*; MIC_{50} =<0.25mg/l and MIC_{90} =1mg/l for *E.faecium*; breakpoint =2mg/l). All the isolates were sensitive to vancomycin (MIC_{50} =4mg/l and MIC_{90} =8mg/l; breakpoint =4mg/l). Some Isolates were resistant to ciprofloxacin (MIC_{50} =4mg/l and MIC_{90} = >256mg/l; range <0.25- >256mg/l; breakpoint =2mg/l for *E.faecalis* and for *E.faecium* MIC_{50} =8mg/l and MIC_{90} = >256mg/l; range 0.5 - >256mg/l; breakpoint =2mg/l). Some isolates were moderately resistant to moxifloxacin (MIC_{50} =4mg/l and MIC_{90} =32mg/l; range <0.25 -64mg/l; breakpoint =4mg/l for *E.faecalis* while *E.faecium* had MIC_{50} =2mg/l and MIC_{90} =32mg/l; range <0.25- 64mg/l; breakpoint =4mg/l). Some isolates were also moderately resistant to amoxicillin (for *E.faecalis* MIC_{50} =2mg/l and MIC_{90} =16mg/l; range 0.5 -32mg/l; breakpoint =4mg/l and *E.faecium* had MIC_{50} =4mg/l and MIC_{90} =32mg/l; range 0.5 -

32mg/l; breakpoint =4mg/l). Some isolates, particularly among the *E.faecium*, were highly resistant to Piperacillin (MIC_{50} = 2mg/l and MIC_{90} =256mg/l; range 1 –256mg/l; Breakpoint =4mg/l). *E.faecalis* were slightly resistant to piperacillin (MIC_{50} =2mg/l and MIC_{90} =8mg/l; range 1 -16mg/l; breakpoint =4mg/l). Some isolates were moderately resistant to augmentin (*E.faecium* had MIC_{50} =4mg/l and MIC_{90} =32mg/l; range 0.5 - 64mg/l; breakpoint =4mg/l and *E.faecalis* had MIC_{50} =4mg/l and MIC_{90} =8mg/l; range 0.5-32mg/l; breakpoint =4mg/l). Some isolates appeared to be highly resistant to tazocin (*E.faecalis* MIC_{50} =4mg/l and MIC_{90} =16mg/l; range 1 –32mg/l; breakpoint =4mg/l. For *E.faecium* MIC_{50} =4mg/l and MIC_{90} =256mg/l; range 4->256mg/l; breakpoint =4mg/l). All isolates were found to be sensitive to linezolid (Zyvox) (MIC_{50} =2mg/l and MIC_{90} =4mg/l; breakpoint =2mg/l). All isolates were moderately resistant to synercid (MIC_{50} =8mg/l and MIC_{90} =32mg/l; range 4 –64mg/l; breakpoint =2mg/l for *E.faecium* and MIC_{50} =16mg/l and MIC_{90} =64mg/l; range 4 –128mg/l; breakpoint =2mg/l for *E.faecalis*.

4.1.2 Nitrocefin testing for β-lactamase production by Isolates

Resistance to β-lactam drugs is a major problem and the results show some resistance in these populations [Fig 4.1(a) and (b)]. It has also been found that some *E.faecalis* strains with high-level gentamicin resistance were also β-lactamase producers (Hindes *et al,*1989; Patterson *et al*, 1988). Therefore it was necessary to test the isolates by using nitrocefin. Nitrocefin is yellow normally but when the β-lactam ring is hydrolysed, it turns red. However, since nitrocefin is a substrate that can be readily hydrolysed by β-lactamase, it provides a sensitive method of detecting β-lactamase. Based on the MICs results of susceptibility test in section 4.1.1, 81 isolates were tested against β-lactamase production and was found that none of the isolates was a β-lactamase producer.

4.2 Antimicrobial Combinations and Synergism *In Vitro* agar method

The effect of *in vitro* antimicrobial agents combinations against *Enterococcus* isolates was investigated by the agar dilution method with combinations of two drugs in attempts to enhance biological effects. There is also a general concept that by employment of the two drugs, it allows lower doses of each drug to be used so that undesirable toxic side effects would be reduced . Alternatively, the combinations could take advantage of the separate effects of each either by the spread of onset of one drug and the prolonged duration of the other or simply to overcome resistance. The emphasis of combinations is not only to enchance biological effects (Synergy) but also to use in the treatment of serious infections such as meningitis before laboratory test and in the mixed infections (Jawetz *et al*, 1952; Moellering *et al*, 1971; Simmons, 1975; Berenbaum, 1978; Rahal, 1978; Lambert *et al*, 2003). The use of antimicrobial combinations to achieve *in vitro* activity and clinical efficacy against organisms resistant to drugs continues to be the subject of intensive investigation and is still a matter of great clinical debate. It should also be noted that certain combinations of agents may yield antagonistic effects (Lepper and Dowling,1951; Moellering,1983) The combinations of some antimicrobial agents (Table 2.2) were tested against all isolates to establish possible synergism. The results are shown as at $MICs_{50}$ and $MICs_{90}$ { Table 4.2 (a) and (b); Fig 4.2 (a) & (b)

4.2.1 Combination of β-lactam with Aminoglycoside by agar method

The combination of amoxicillin at a fixed concentration of 16mg/l each in each of the serial two-fold dilution of gentamicin ranging 0.25-256mg/l was based on the method in section 2.2.5.1 and was done in order to establish synergistic activity of the combined

drugs against 81 isolates. The results show that there was more than four-fold reduction in the MIC of the combined drugs as compared with the MIC of the single drug alone [Table 4.1 (a) & (b); Fig 4.2 (a), (b)]. The original observation that penicillin combined with aminoglycoside (Streptomycin) resulted into synergism was in 1947 (Hunter, 1947). However, several investigators have demonstrated a synergistic effect of penicillin or other β-lactam drugs to various aminoglycosides including gentamicin (Calderwood *et al*,1977; Moellering *et al,* 1971; Weinstein and Lentek,1976). In this study, the combination of amoxicillin and gentamicin revealed the four-fold reduction in the MIC of the combined drugs showing the synergistic activity of the two drugs and confirmed the findings of other investigators (Basker and Sutherland, 1977; Russell and Sutherland, 1975).

4.2.2 Combination of Aminoglycoside with Glycopeptide by agar method

Vancomycin at a fixed concentration of 4mg/l each was combined with each of the serial two-fold dilution of gentamicin ranging 0.25-256mg/l using the method in section 2.2.5.1 to show the synergistic activity of the combined drugs against 81 isolates. The results indicate that there was more than 4-fold reduction in the MIC of the combined drugs compared with MIC of single antibiotic alone (Table 4.1 (a) & (b)). Vancomycin is a cell-wall active agent which can enhance the entry of aminoglycoside such as gentamicin into enterococcal isolates (Moellering and Weinberg, 1971) resulting in production of synergism when combined with aminoglycoside. The four-fold reduction in the MIC of the combined drugs showed the synergistic activity against the isolates.

4.2.3 Combination of Streptogramins and Glycopeptides by agar method

4.2.3.1 combination of Teicoplanin and Synercid

Teicoplanin at a fixed concentration of 2mg/l each against *E.faecalis* and 0.5mg/l each against *E.faecium* was combined with each of the serial two-dilution of synercid based on the method in section 2.2.5.1 in order to demonstrate the synergistic activity of the combined drugs against 81 isolates. Since *E.faecalis* is more resistant to synercid (MIC range 4-128mg/l) than *E.faecium* (MIC range 4-64), different fixed concentrations of teicoplanin indicated above had been selected for the test based on the sensitivity of both species against the teicoplanin. The results showed reduction of more than 4-fold in the MIC of the combined drugs as compared with MIC of single antibiotic alone [Fig 4.2 (a) & (b) Table 4.1 (a) & (b)].Teicoplanin is a cell-wall active drug; while synercid binds to the 50s ribosomal subunit resulting in inhibition of protein synthesis. Perhaps teicoplanin also enhances the entry of synercid into bacterial cell-wall; thus, the combined action of the two drugs resulted into synergy. Hill *et al,* 1997, established the synergistic activity of the two drugs which resulted into the reduction of more than 4-fold in the MIC of the combined drugs against isolates. This finding agreed with the result of this study with respect to the reduction of more than four-fold in the MIC of the combined drugs in line with others findings indicating the synergistic activity of the two drugs.

4.2.3.11 Combination of Synercid and Vancomycin

The combination of vancomycin at a fixed concentration of 4mg/l each with each of two-fold serial dilution of synercid range 0.25-256mg/l to establish the synergistic activity of

the combined drugs against 81 isolates using the method in section 2.2.5.1. The results showed no or little reduction in MIC of the combined drugs as compared with MIC of single drug alone. Vancomycin is a cell-wall active agent; while synercid acts on 50s ribosomal subunit resulting in inhibition of protein synthesis. Hill *et al*, 1997 could not find the synergistic activity of the combination of the two drugs since there was no reduction in MIC of the combined drugs significantly. These findings agreed with the results of this study which found no significant reduction in the MIC of the combined drugs. This is an indication of antagonistic activity of the two combined drugs

4.2.4 Combination of Fluoroquinolone and Glycopeptide by agar method

Teicoplanin at a fixed concentration of 4mg/l each was combined with each of two-fold serial dilution of ciprofloxacin range 4-256mg/l. The test did not include the sensitive isolates against ciprofloxacin with MICs range of <0.25-2mg/l because they were considered sensitive against the ciprofloxacin. The method used in the test was indicated in section 2.2.5.1 to demonstrate the synergistic activity against isolates. The results showed reduction of more than 4-fold in the MIC of the combined drugs as compared with single antibiotic alone (Fig 4.2 (a) & (b); Table 4.1 (a) & (b). Teicoplanin acts on the cell wall of the bacteria; while ciprofloxacin inhibits bacterial activity by interacting with type II topoisomerase (*gyr*A) and topoisomerase IV (*par*C) (Shen *et al,*1989) of the bacterial DNA; this combination results into synergy. The combination of the two drugs in this study resulted in more than four-fold reduction in the MIC of the combined drugs indicating the synergistic activity of both drugs

4.2.5 Combination of Streptogramins and β-lactam by agar method

Amoxicillin at a fixed concentration of 4mg/l each against *E.faecalis* and 2mg/l each against *E.faecium* was combined with each of the serial two-fold dilution of synercid

range 4-128mg/l based on the method in section 2.2.5.1 to show the synergistic activity of the combined drugs against isolates. Different fixed concentrations as above for synercid were selected based on the sensitivities of the isolates (*E.faecalis* MIC range 4-128mg/l and *E.faecium* range 4-64mg/l) against the drug. The results showed that there was a 4-fold reduction in the MIC of the combined drugs as compared to MIC of single drug alone (Table 4.1 (a) & (b) synercid binds to the 50s ribosomal subunit of the bacterial DNA resulting into inhibition of protein synthesis. In this study, the combination of both drugs resulted in the reduction of the MIC of the combined drugs of more than four-fold . This result shows the synergistic activity of both drugs

4.2.6 Combination Fluoroquinolone and Streptogramins by agar method

Synercid at a fixed concentration of 8mg/l each was combined with each of the serial two-fold dilution of ciprofloxacin range 8-256mg/l against *E.faecium* based on the method in section 2.2.5.1 to demonstrate the synergistic activity of the combined drugs against isolates. Isolates sensitive to ciprofloxacin range <0.25-4mg/l were excluded because of their sensitivity against the ciprofloxacin. The results showed the reduction of 4-fold in the MIC of the combined antibiotics as compared with the drug alone (Table 4.1 (a) & (b)). *E.faecalis* isolates were not tested against the combined drugs because *E.faecalis* is intrinsically resistant to synercid. Ciprofloxacin acts on both *gyr*A and *par*C of the bacterial DNA and synercid binds to 50s ribosomal subunit of the bacterial DNA and inhibits protein synthesis. The combination of both drugs by Hill *et al*, 1997 resulted into synergism. A similar combination of both drugs against 26 *E.faecium* isolates in this study resulted in more than a four-fold reduction in the MIC of the combined drugs. This indicates synergistic activity of both drugs, thus agreeing with the findings by Hill *et al*, 1997.

Table 4.2 Combined Antibiotics at MIC_{50} and MIC_{90} for *E.faecalis* and *E.faecium*

(a) *Enterococcus faecalis*

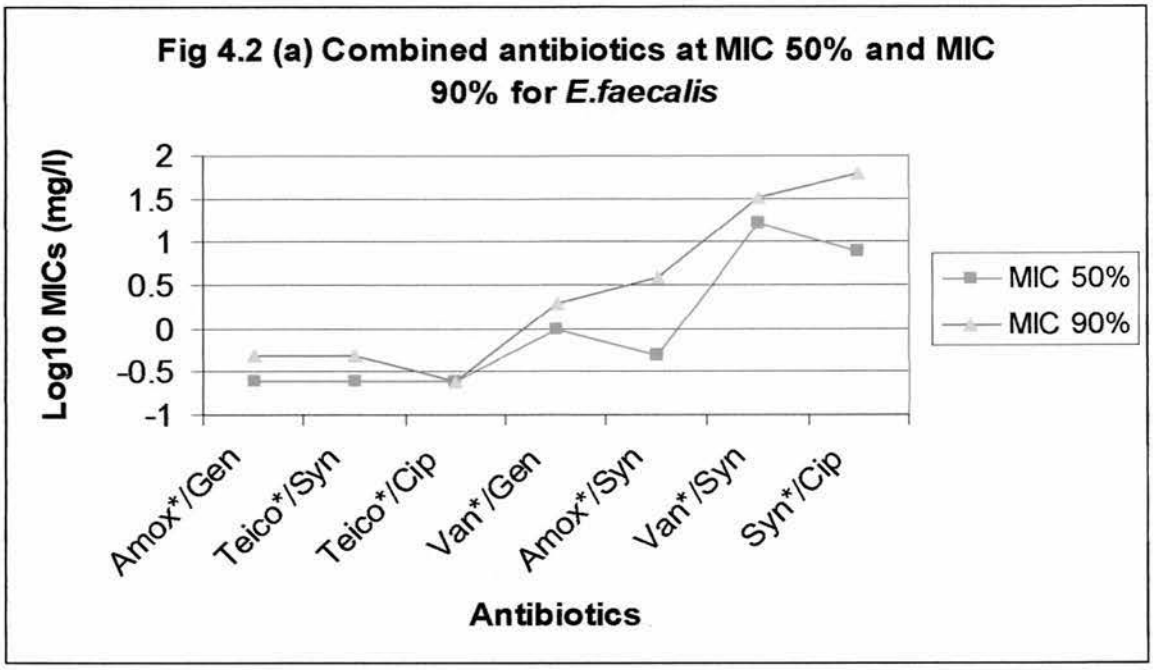
Antibiotic	MIC Range (mg/l)	MIC_{50}	MIC_{90}
Amox*/Gent	<0.25-1	<0.25	0.5
Teico*/Syn	<0.25-0.5	<0.25	0.5
Teico*/Cip	<0.25-<0.25	<0.25	<0.25
Vanc*/Gent	<0.25-32	1	2
Amox*/Syn	<0.25-16	0.5	4
Vanc*/Syn	8-64	16	32
Syn*/ Cip	1-64	8	64

*=Fixed Concentration of Antibiotic (Amox=16mg/l, Teic=2mg/l, Van=4mg/l).

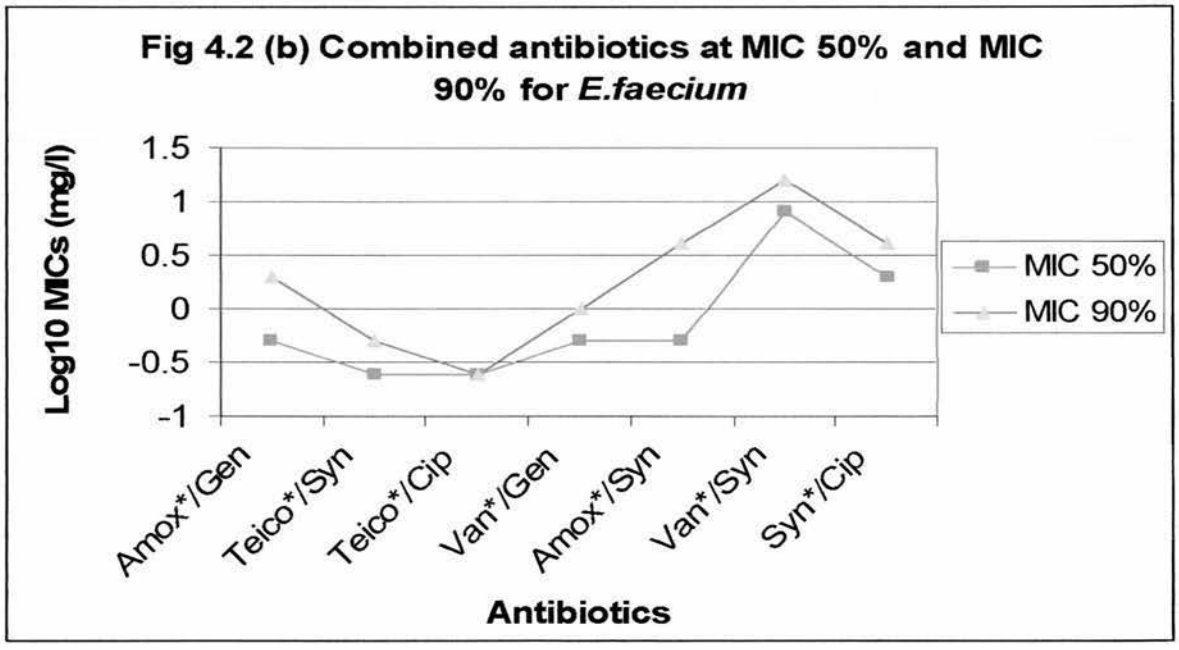
(b) *Enterococcus faecium*

Antibiotic	MIC Range (mg/l)	MIC_{50}	MIC_{90}
Amox*/Gent	<0.25-4	0.5	2
Teico*/Syn	<0.25-0.5	<0.25	0.5
Teico*/Cip	<0.25-<0.25	<0.25	<0.25
Vanc*/Gent	<0.25-2	0.5	1
Amox*/Syn	<0.25-8	0.5	4
Vanc*/Syn	4-64	8	16
Syn*/ Cip	<0.25-8	2	4

* = Fixed concentration of Antibiotics (Amox=16mg/l, Teic=0.5mg/l, van=4mg/l, Syn=8mg/l).



* =Fixed antibiotic concentration



* =Fixed antibiotic concentration

4.3 Synergy testing of the combined antimicrobial agents by Checkerboard method

The Checkerboard technique is probably the test most frequently used for analysis of interaction between the antibiotics (Holm, 1986). Beerenbaum in 1978, proposed a mathematical way to describe the results of checkerboard test which he had called fractional inhibition concentrations (FIC) index. He gave the value of FIC index of 0.5 to mean synergism while ≥ 1 would indicate additive or indifferent effect of the combined drugs and >2 would mean antagonism. The FIC method allows a more objective analysis of the result than the mere drawing of isobologram lines and therefore widely used (Holm,1986). The selection of the isolates used in this investigation was based on the fact that all the isolates (*E.faecium* and *E.faecalis*) collected from RIE were sensitve to glycopeptide drugs but resistance to gentamicin and therefore, there was a need to use isolates with resistance to both gentamicin and vancomycin to establish synergy. Thus resulted in getting some isolates with *van*B type of resistance from Dundee for the test. In this study, Owing to the laborious work involves in using the checkerboard agar dilution method to assess synergistic activity of the combined antimicrobial agents against isolates, only three strains and the control NCTC12202 of vancomycin-resistant *E.faecium* were tested against the combination of ciprofloxacin and synercid. It involved measuring the MIC of each drug for each isolate by doubling agar dilution and then the known MIC for each drug was fine-tuned by re-measuring in an arithmetical progression. The MIC was taken as fractional inhibitory concentration (FIC) of 1.0 for each antibiotic. Agar plates containing mixture of both drugs were made up. The plates were inoculated

with isolates using a multiple inoculating device (Denley A400) yielding final inocula of approximately 10^4 CFU per spot. The plates were examined for growth or non growth after 24 hours of incubation at 37°C. The results showed isolates had the following FIC indices: D002= 0.4, G051= 0.4, 788/5/905=0.3 and NCTC12202= 0.3 (see also Figs 4.3 , 4.4, 4.5, 4.6 and Table 4.3). These results are in complete agreement with what Beerenbaum had proposed in 1978 with respect to synergism. The results confirm the existence of synergistic activity of the two drugs against four strains of *E.faecium*. However, the correlation between the FIC indices of these unrelated strains and the *in vitro* agar dilution data suggest that synergy between the two combined drugs might be common in enterococci. The clinical efficacy of the combinations and the contribution of the synergistic activity have yet to be proven clinically.

Table 4.3 FIC of Checkerboard and Types of Resistance-*E.faecium*

Isolate #	FIC	Types of Resistance
D002	0.4	*Van*B
G051	0.4	*Van*B
788/5/95	0.3	*Van*B
NCTC12202	0.3	Sensitive

Fig 4.3 Fractional Inhibitory Concentration for *E.faecium* D002

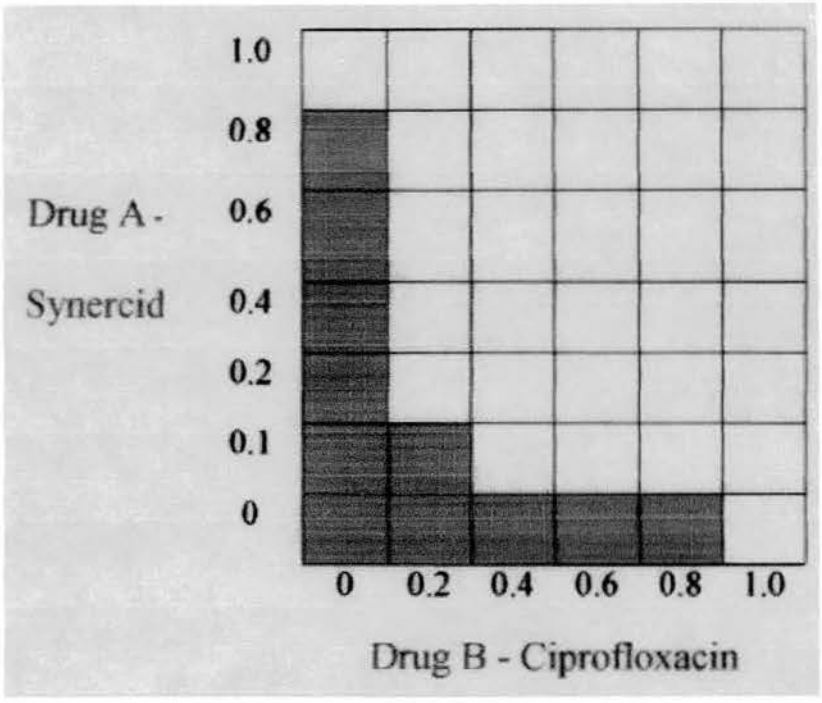


Fig 4.4 Fractional Inhibitory Concentration for *E.faecium* G051

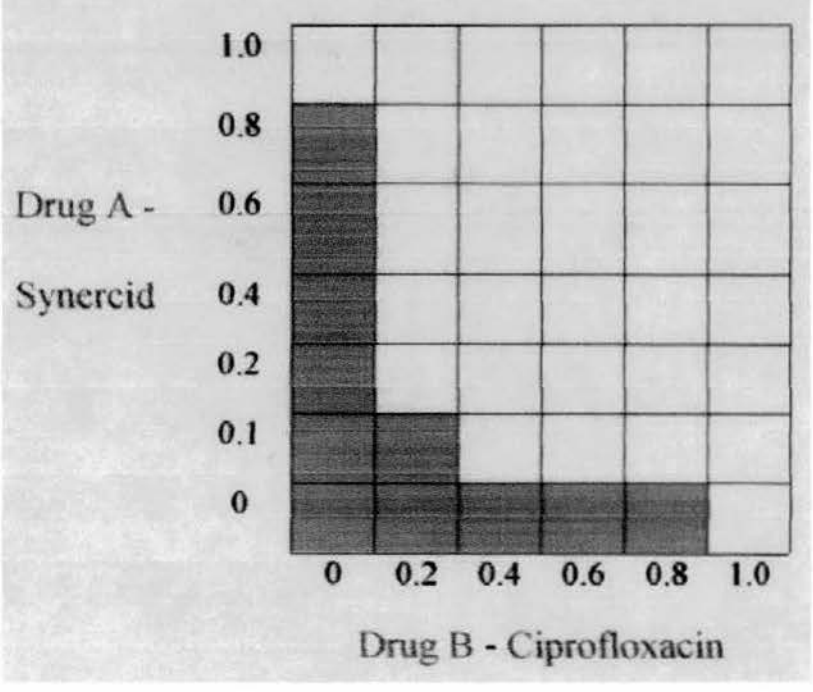


Fig 4.5 Fractional Inhibitory Concentration for *E.faecium* 788/5/95

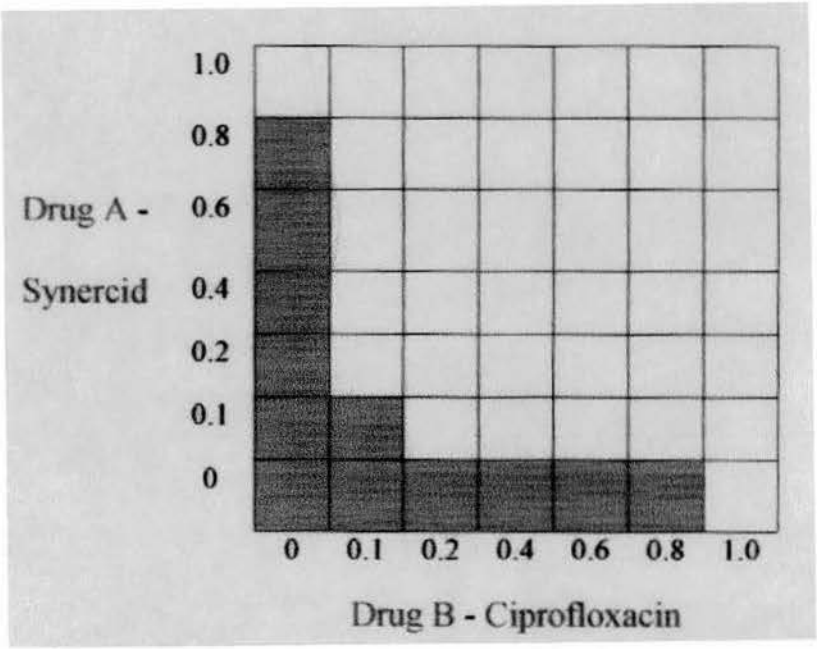
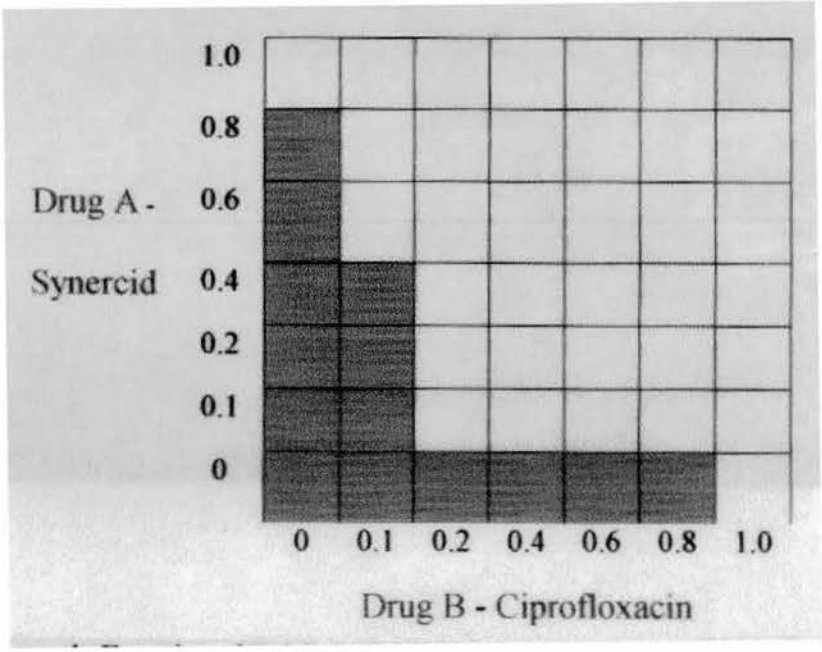


Fig 4.6 Fractional Inhibitory Concentration for *E.faecium* NCTC 12202



4.4 Synergy testing of the combined antimicrobial agents by Time-Kill Curves method.

The time-kill curves technique measures the microbicidal activity of the combination being tested. It is presumably more relevant for clinical situations in which bactericidal therapy is desirable. It also provides a dynamic picture of antimicrobial action and interaction over time based on serial colony counts. However, the procedure is tedious and laborious. The selection of the isolates (17 *E.faecium* and 22 *E.faecalis*) used in the investigation was based on their MICs {Table 4.4 (a) & (b) } and also limited financial resources available. The experiment was performed with a final inoculum of 10^5 to 10^7 cfu/ml produced by diluting an overnight culture growth of each isolate in BHI broth and adjusting to logarithmic-phase culture; then diluted a second time in fresh BHI broth and added appropriate amounts of antimicrobial agent(s) in each tube. The initial sampling for colony counts was done as soon as antimicrobial was added in each tube by removing 0.1ml from each tube and diluting with saline in series of marked tubes (10^{-1}, 10^{-2}, $10^{-3}$and 10^{-4}) to make 10 fold dilution for each tube. Using the sterile pipette, dropped approximately 20µl of each 10 fold dilution in each corner of a triangle marked on the bottom of each BHIA plate (i.e a total of three drops per triangle for each concentration of 10 fold dilutions for a single sterile pipette). The plates were incubated at 37°C for 24-48 hours. Colony counts was done based on the colony growth shown in Fig 4.7 (e.g at concentration of 10^{-4} dilution for Control Isolate at 8 hours). While Fig 4.8 showed no growth for the combined antibiotics (eg, gentamicin/amoxicillin) at 24 hours. Time-kill curves $\log_{10}$ graphs for 22 *E.faecalis* and 17 *E.faecium* with gentamicin, synercid, vancomycin, teicoplanin, ciprofloxacin and amoxicillin in various combinations including alone are shown in Figs 4.9-4.32 and appendix B. Bactericidal synergy was

defined as the killing of at least 2 $\log_{10}$ CFU/ml in comparison with most active single drug. In this study, There was a 2 log decrease at 8 hour of the combined amoxicillin (8mg/l) and gentamicin (64mg/l) against isolates (17B/235, 17B/721 and 18B/234-*E.faecium*) and isolates (17B/407 and 18B/309-*E.faecalis*) indicatng synergistic activity of the combined antibiotics (Figs 4.9--4.13). However, regrowth occurred as shown in Figs 4.9, 4.11 and 4.13 at 24 hour for amoxicillin alone. When combined teicoplanin (2mg/l) and synercid (64mg/l) against isolate (18B/254-*E.faecium*), there occurred a 2 $\log_{10}$ decrease at 8 hour and a complete killing of bacteria at 24hour (Fig 4.14) indicating synergistic activity. Teicoplanin alone had regrowth at 24 hour (Fig 4.14). The combination of teicoplanin (2mg/l) with synercid (64mg/l) against isolate (17B/686-*E.faecalis*) resulted in a decrease of 2 log at 8 hour and the occurrence of complete killing of bacteria at 24 hour (Fig 4.15) and regrowth of teicoplanin at 24 hour (Fig 4.15). The combination of synercid (32mg/l) and amoxicillin (8mg/l) against isolate (18B/960-*E.faecium*) resulted in a decrease of 2 log at 8 hour and a complete killing of bacteria at 24hour (Fig 4.16). There was regrowth of amoxicillin at 24 hour. Since isolate (19B/391-*E.faecium*) had MICs for both synercid and amoxicillin at 64 mg/l, test was made first at 32mg/l for synercid combined with amoxicillin (8mg/l) resulting in a 2 log decrease CFU/ml and a complete killing of bacteria at 24 hour (Fig 4.17) and regrowth occurred at 24 hour. Combination of synercid (64mg/l) with amoxicillin (8mg/l) against isolate (18B/376-*E.faecalis*) resulted in a 2 log decrease at 8 hour and a complete isolate being killed at 24 hour (Fig 4.18). Regrowth of amoxicillin occurred at 24 hour. Since isolate (18B/309-*E.faecalis*) had MICs of 32 mg/l for each amoxicillin and synercid, test was first done with combination of synercid (16mg/l) with amoxicillin (8mg/l) which resulted in a 2 log decrease in CFU/ml at 8hour (Fig 4.19). Then the reverse was done (i.e 16mg/l for amoxicillin and 8mg/l for synercid) but there was not much difference.

Ciprofloxacin (64mg/l) combined with synercid (8mg/l) against isolate (18B/294-*E.faecium*) had 2 log decrease at 8hour and a complete killing of the bacteria at 24 hour (Fig 4.20) and regrowth occurred at 24 hour. Similarly, when ciprofloxacin (64mg/l) was combined with synercid (16mg/l) against isolate (18B/517-*E.faecium*), a 2 log decrease occurred at 8 hour with a complete killing of bacteria at 24 hour (Fig 4.21) and regrowth of synercid occurred at 24 hour. Combination of ciprofloxacin (64mg/l) with synercid (8mg/l) against isolate (18B/414-*E.faecalis*) resulted in the decrease of 2 $\log_{10}$ at 8 hour and a complete killing of bacteria at 24 hour (Fig 4.22). Regrowth for synercid occurred at 24 hour. When ciprofloxacin (4mg/l) was combined with synercid (32mg/l) against isolate (18B/745-*E.faecalis*), a 2 log decrease occurred at 8 hour with a complete bacterial death at 24 hour (Fig 4.23). Combination of vancomycin (8mg/l) with gentamicin (64mg/l) against isolates (18B/314 and 18B/578-*E.faecalis*) resulted in 2 log decrease for each combination at 8 hour and a complete killing of bacteria at 24 hour (Figs 4.24 and 4.25 respectively) and regrowth occurred at 24 hour for vancomycin (Fig 4.25). When vancomycin (64mg/l) was combined with gentamicin (16mg/l) against isolate (G051-*E.faecium*), a 2 log decrease occurred at 8 hour with complete killing of the bacteria at 24 hour (Fig 4.26). Combination of gentamicin (64mg/l) with vancomycin (4mg/l) against isolate (18B/33-*E.faecium*) resulted in a 2 log decrease with a complete killing of bacteria at 24 hour (Fig 4.27). Combination of teicoplanin (2mg/l) with ciprofloxacin (64mg/l) against isolate (19B/471-*E.faecium*) resulted in a 2 log decrease at 8 hour and a complete killing of bacteria occurred at 24 hour (Fig 4.28). Similarly, combination of teicoplanin (2mg/l) with ciprofloxacin (64mg/l) against isolate (19B/412-*E.faecalis*) resulted in a decrease of 2 $\log_{10}$ at 8 hour and a complete death of bacteria occurred at 24 hour (Fig 4.29) and regrowth in teicoplanin occurred at 24 hour. However, combination of vancomycin (8mg/l) with synercid (64mg/l) against isolates

(18B/487 and 18B/506-*E.faecium*) resulted in a 2 log increase for each combination at 8 hour. This indicated antagonistic activity of the two antibiotics (Figs 4.30 and 4.31 respectively) and regrowth occurred at 24 hour for vancomycin. When vancomycin (4mg/l) was combined with synercid (16mg/l) against isolate (18B/791-*E.faecalis*), there was also increase of 2 logs at 8hour (Fig 4.32). Again, it indicated antagonism of the antibiotic combinations and regrowth occurred at 24 hour for vancomycin.

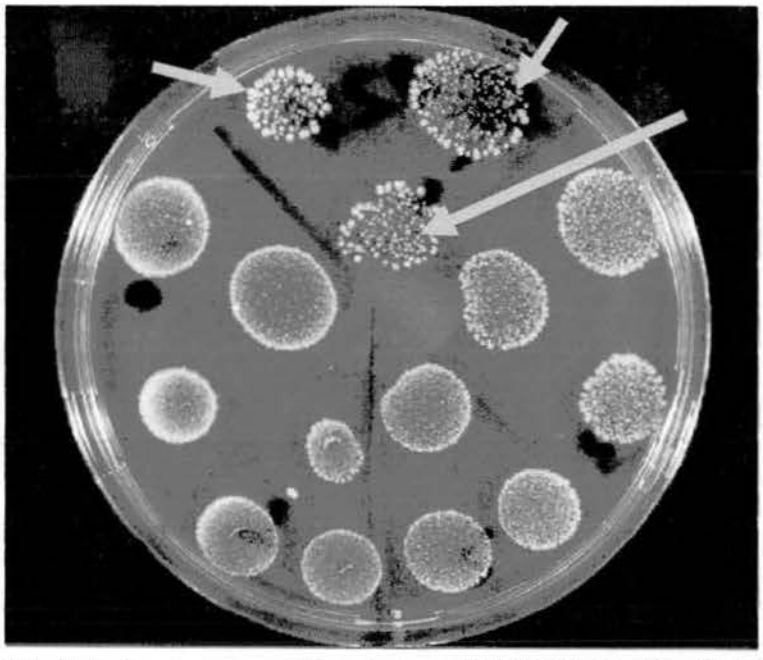


Fig 4.7 Arrangement for drops of 10 fold dilution in triangles marked and colony growth on BHIA plate at 24 hour indicated by Yellow arrows in a triangle for counting CFU/ml

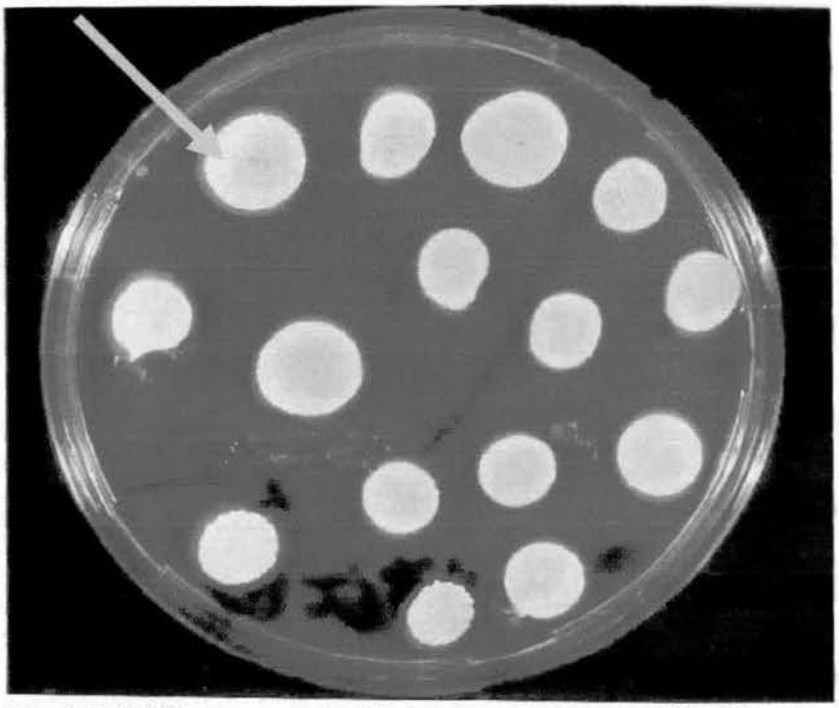


Fig 4.8. Yellow arrow indicates no colony growth at 24 hours for combined antibiotics (eg Gen/Amo)

Table 4.4 (a) & (b) MICs (mg/l) for enterococci (*E.faecalis* & *E.faecium*) used in Time-kill Curve Technique

(a) 22 *E.faecalis*

Isolate #	Genta	Syner	Vanco	Teico	Cipro	Amoxi
17B/407	>256	64	8	1	>256	1
17B/452	>256	32	4	1	>256	1
17B/686	>256	128	8	0.5	>256	1
17B/761	64	64	4	1	>256	4
17B/819	128	64	4	<0.25	>256	4
18B/89	256	128	4	2	>256	4
18B/309	128	32	8	1	0.5	32
18B/329	64	32	4	1	0.5	4
18B/337	>256	128	8	1	>256	4
18B/358	>256	128	8	1	>256	2
18B/376	>256	128	8	1	>256	0.5
18B/379	>256	128	8	1	>256	4
18B/382	64	32	8	1	0.5	2
18B/387	64	32	8	1	0.5	0.5
18B/390	>256	64	4	1	>256	4
18B/414	>256	128	4	1	>256	4
18B/506	>256	128	4	1	>256	4
18B/555	128	64	4	1	0.5	4
18B/578	>256	64	4	0.5	>256	1
18B/745	64	64	8	0.5	0.5	0.5
18B/791	64	32	4	<0.25	0.5	1
19B/412	128	64	4	0.5	>256	4

Genta= gentamicin, Syner= synercid, Vanco= vancomycin, Teico= teicoplanin, Cipro=ciprofloxacin and Amoxi= amoxicillin.

(b) 17 *E.faecium*

Isolate #	Genta	Syner	Vanco	Teico	Cipro	Amoxi
17B/235	>256	8	4	1	>256	16
17B/721	256	16	4	1	64	1
17B/849	>256	8	4	0.5	0.5	16
18B/33	>256	8	4	1	0.5	16
18B/234	256	16	8	1	>256	64
18B/254	>256	128	8	1	>256	0.5
18B/294	>256	32	8	1	>256	32
18B/298	128	32	4	1	0.5	4
18B/487	>256	128	8	1	8	32
18B/517	256	8	8	1	>256	32
18B/632	256	8	4	1	>256	32
18B/749	>256	4	4	2	0.5	1
18B/960	>256	64	4	<0.25	>256	1
19B/391	256	64	4	0.5	>256	64
19B/471	>256	64	4	0.5	>256	1
D002	32	4	32	2	16	8
G051	32	4	>256	2	8	16

Genta= gentamicin, Syner= synercid, Vanco= vancomycin, Teico= teicoplanin, Cipro= ciprofloxacin and Amoxi= amoxicillin

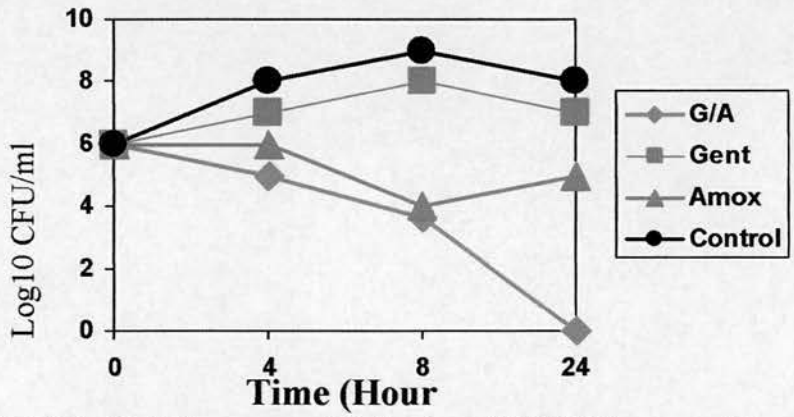


Fig 4.9 Kinetic kill curves of *E.faecium* (17B/235) exposed to gentamicin (64mg/l) combined with amoxicillin (8mg/l)

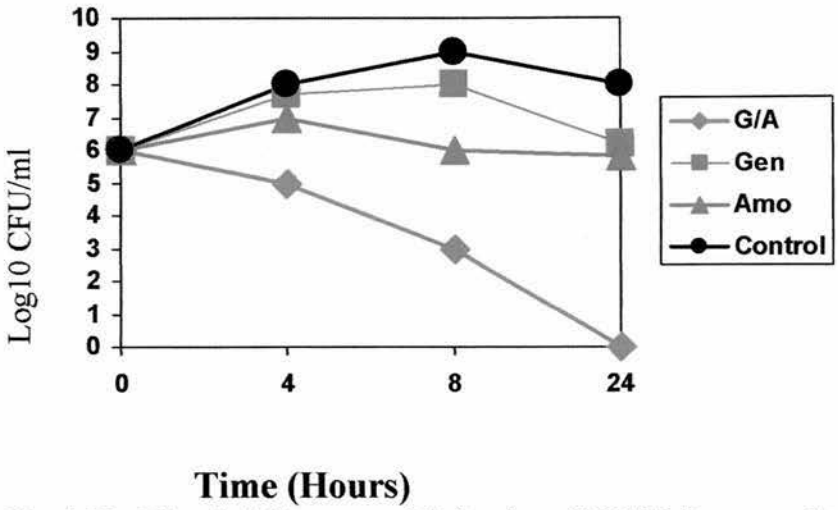


Fig 4.10 Kinetic kill curve of *E.faecium* (18B/234) exposed to gentamicin (64mg/l) combined with amoxicillin (8mg/l)

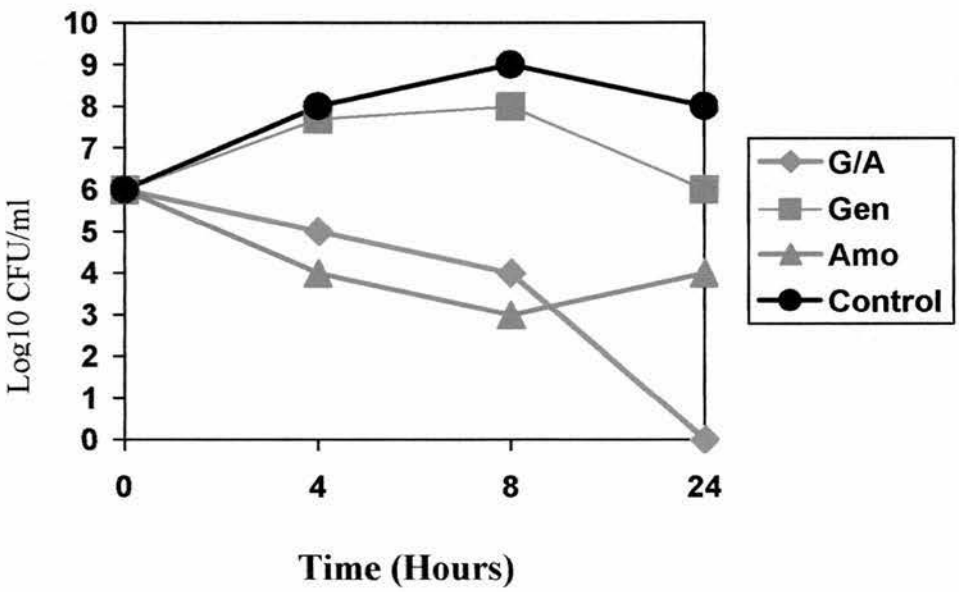


Fig 4.11 Kinetic kill curves of *E.faecium* (17B/721) exposed to gentamicin (64mg/l) combined with amoxicillin (8mg/l)

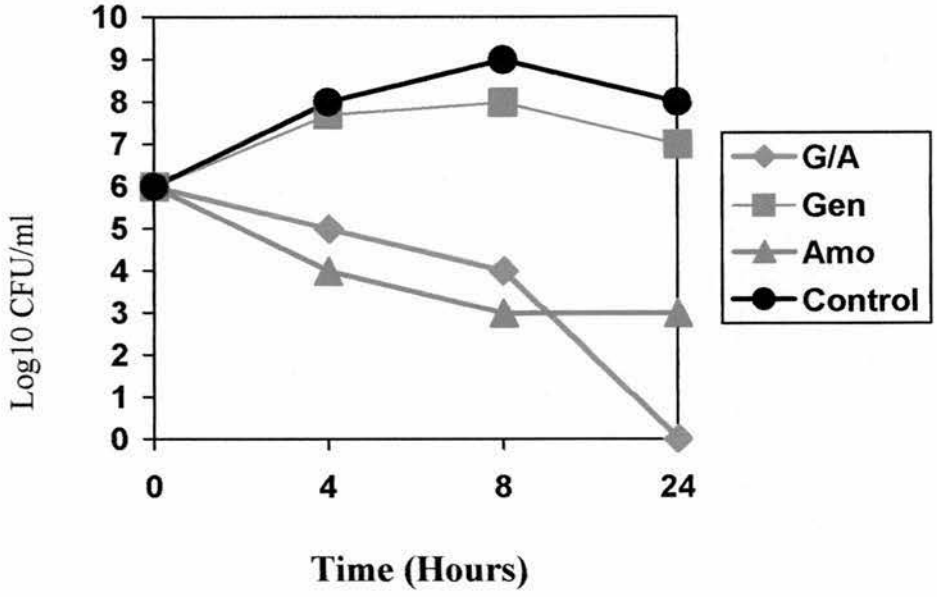


Fig 4.12 Kinetic kill curves of *E.faecalis*(17B/407) exposed to gentamicin (64mg/l) combined with amoxicillin (8mg/l)

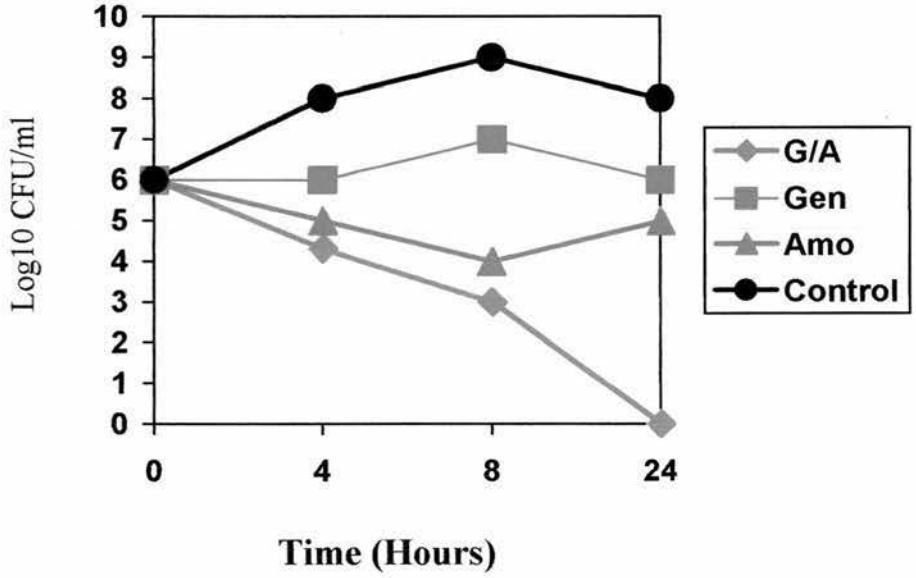


Fig 4.13 Kinetic kill Curves of *E.faecalis* (18B/309) exposed to gentamicin (64mg/l) combined with amoxicillin (8mg/l). G/A=Combined gentamicin/amoxicillin, Gen=gentamicin, Amo=amoxicillin.

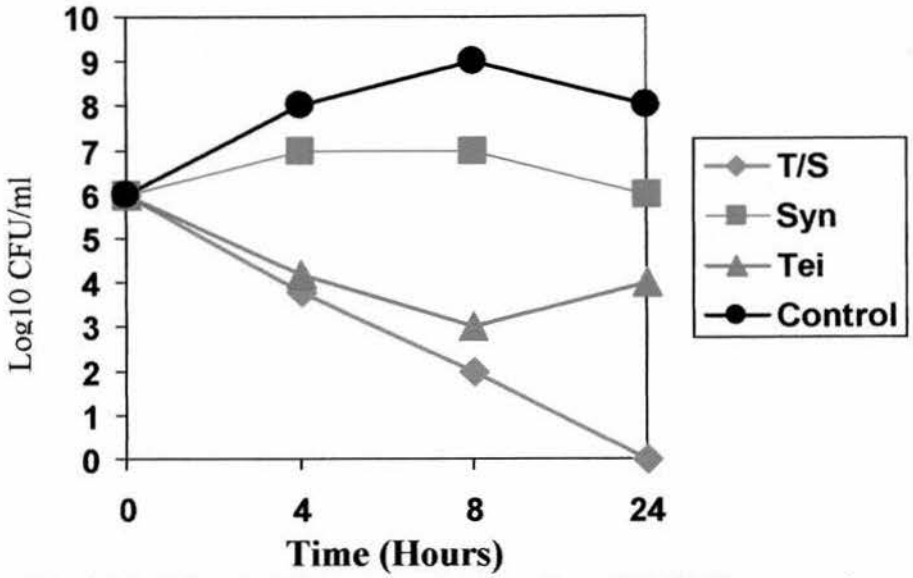


Fig 4.14 Kinetic kill curves of *E.faecium* (18B/254) exposed to synercid (64mg/l) combined with teicoplanin (2mg/l)

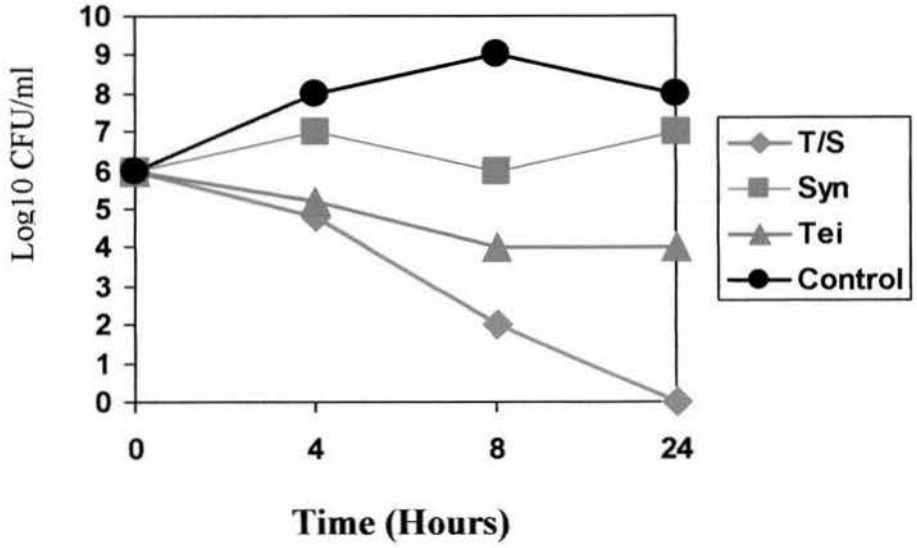


Fig 4.15 Kinetic kill curves of *E.faecalis* (18B/686) exposed to synercid (64mg/l) combined with teicoplanin (2mg/l). T/S=Combined teicoplanin/synercid, Syn=synercid, Tei=teicoplanin.

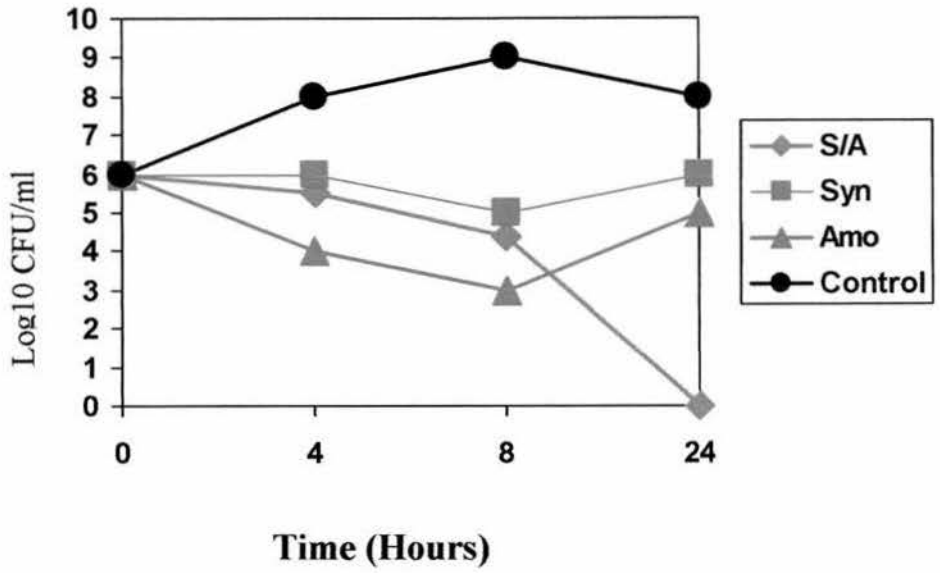


Fig 4.16 Kinetic curves of *E.faecium* (18B/960) exposed to synercid (32mg/l) combined with amoxicillin (8mg/l)

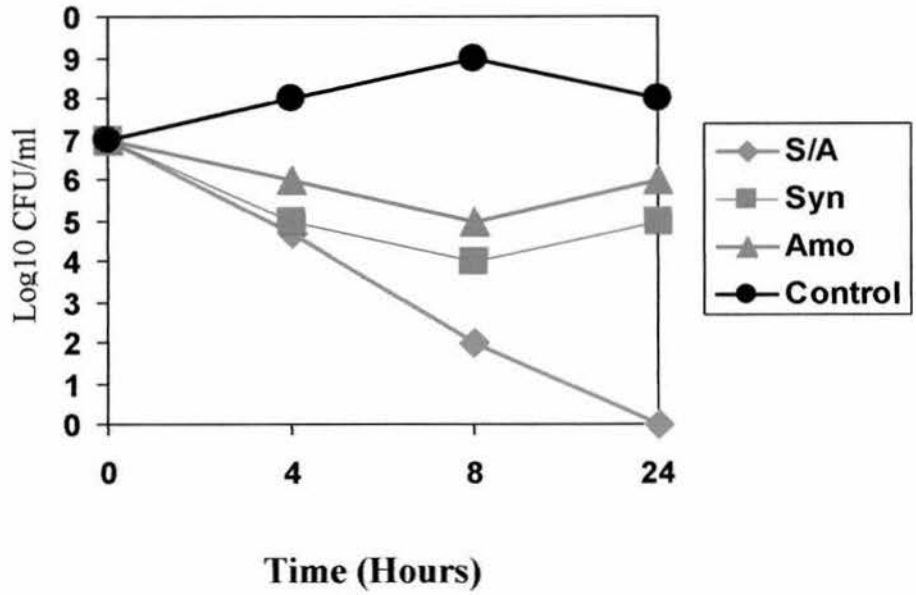


Fig 4.17 Kinetic curves of *E.faecium* (19B/391) exposed to Synercid (32mg/l) combined with amoxicillin (8mg/l)

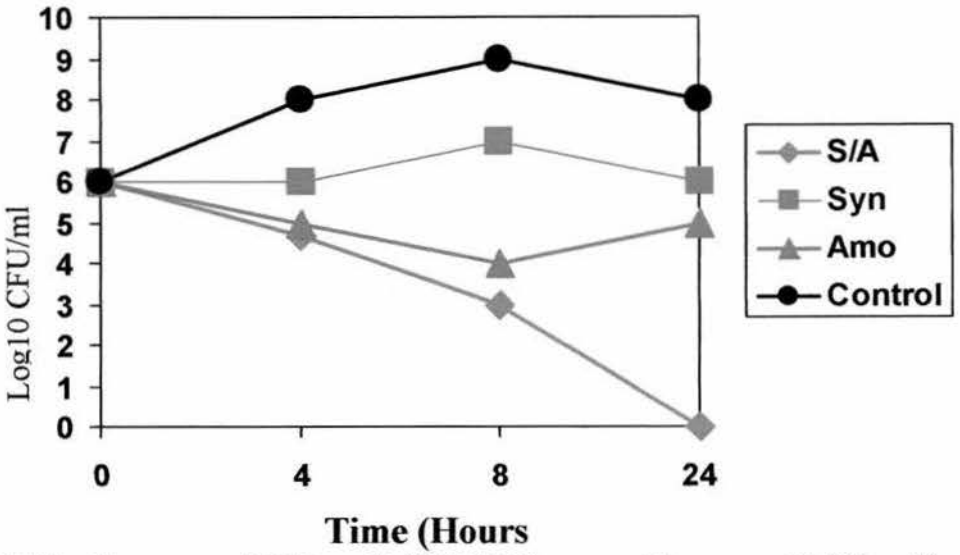


Fig 4.18 Kinetic curves of *E.faecalis* (18B/376) exposed to synercid (64mg/l) combined with amoxicillin (8mg/l

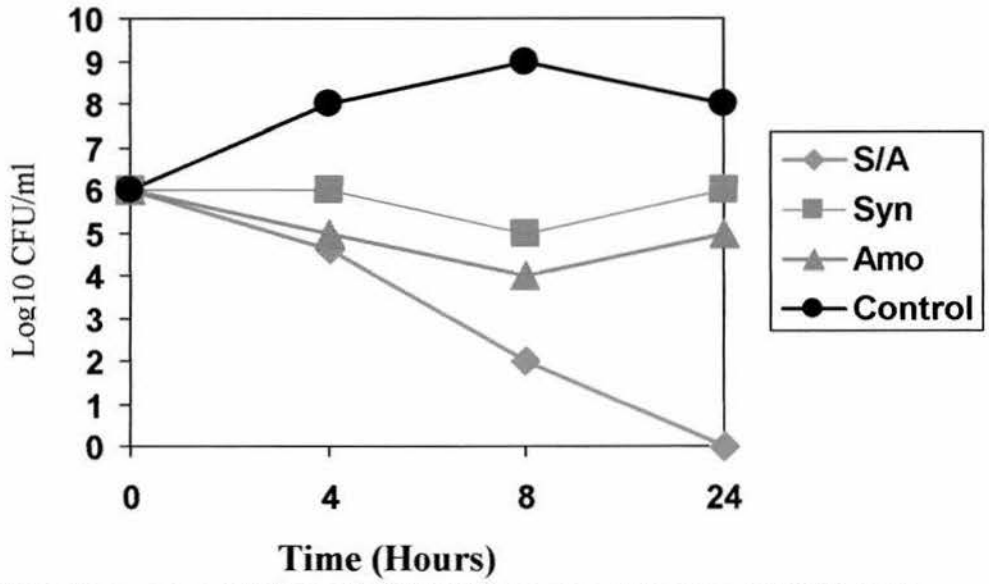


Fig 4.19 kinetic curves of *E.faecalis* (18B/309) exposed to synercid (16mg/l) combined with amoxicillin (8mg/l). S/A=Combined synercid/amoxicillin, Syn=synercid, Amo=amoxicillin.

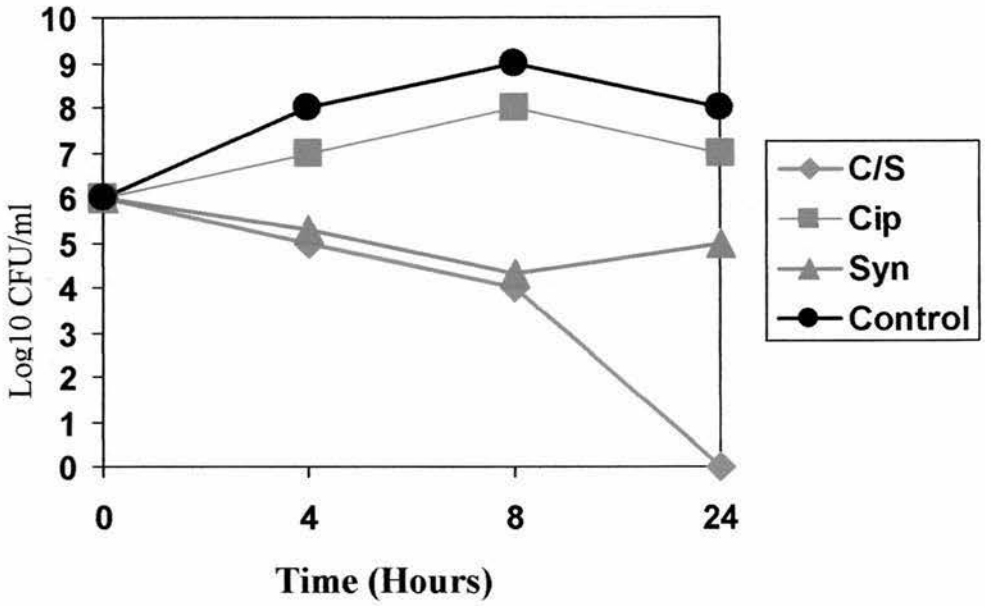


Fig 4.20 Kinetic curves of *E.faecium* (18B/294) exposed to ciprofloxacin (64mg/l) combined with synercid (8mg/l)

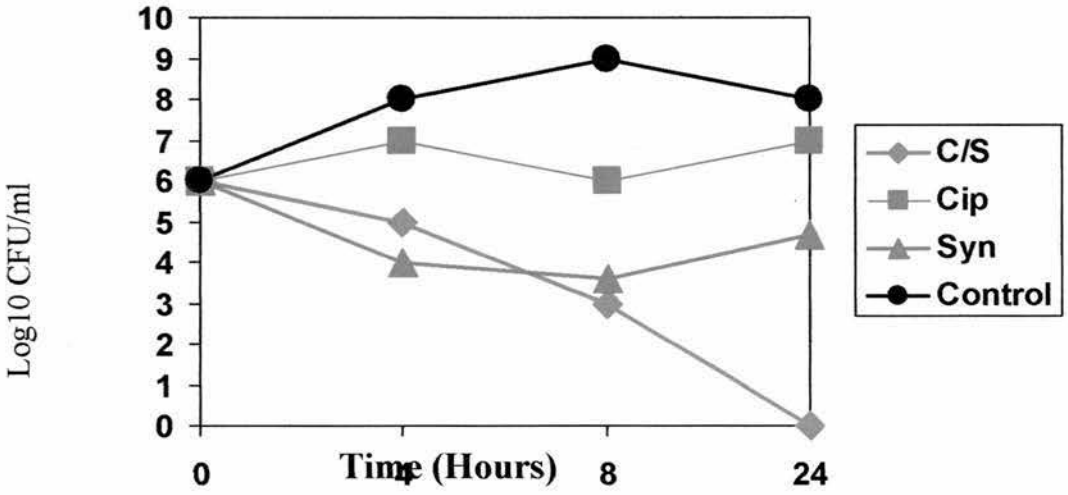


Fig 4.21 Kinetic curves of *E.faecium* (18B/517) exposed to ciprofloxacin (64mg/l) combined with synercid (8mg/l)

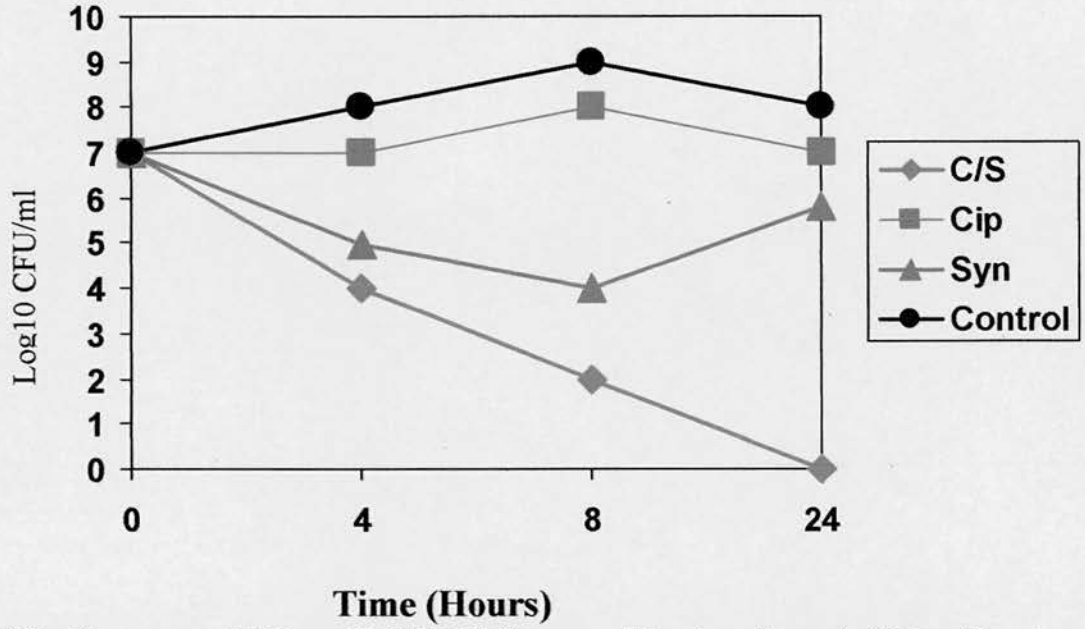


Fig 4.22 Kinetic curves of *E.faecalis* (18B/414) exposed to ciprofloxacin (64mg/l) combined with synercid (16mg/l)

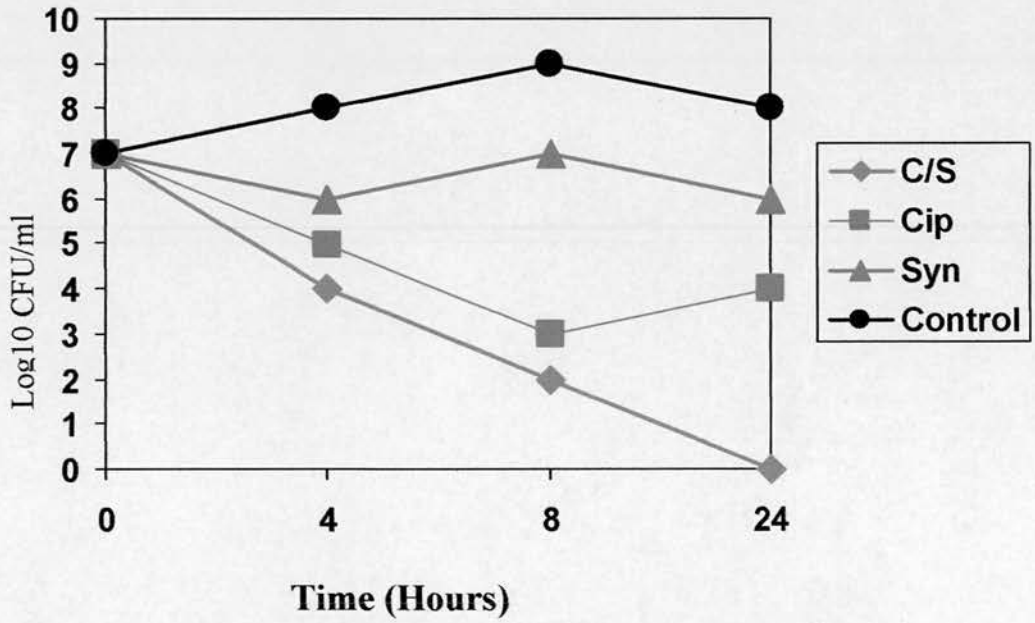


Fig 4.23 Kinetic curves of *E.faecalis* (18B/745) exposed to synercid (32mg/l) combined with ciprofloxacin (4mg/l). C/S=Combined ciprofloxacin/synercid, Cip=ciprofloxacin, Syn=synercid.

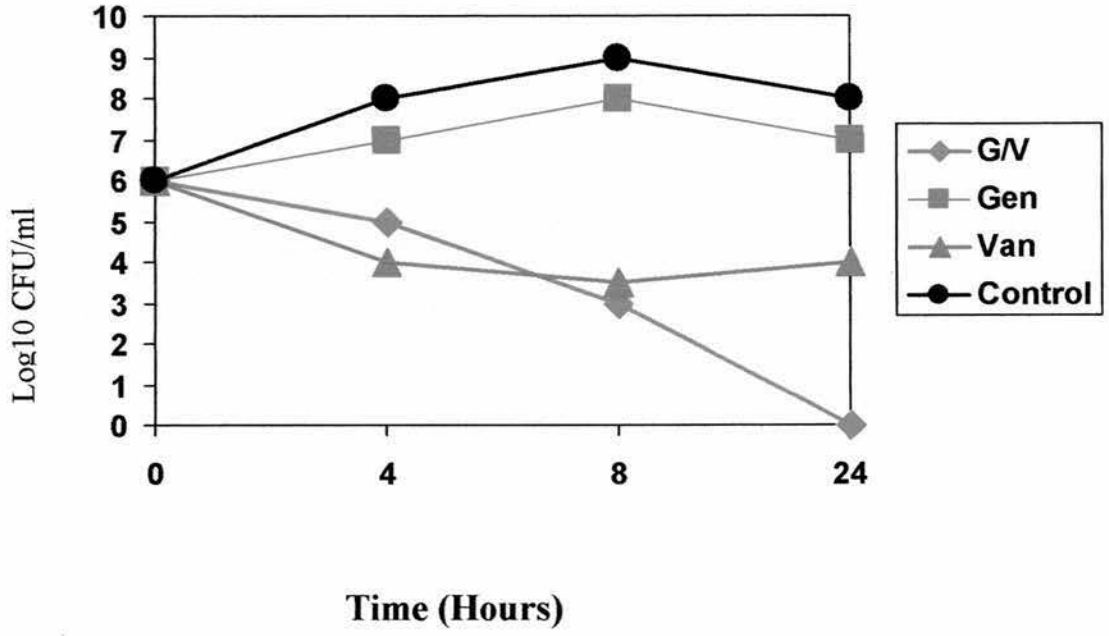


Fig 4.24 Kinetic curves of *E.faecalis* (18B/314) exposed to gentamicin (64mg/l) combined with vancomycin (8mg/l)

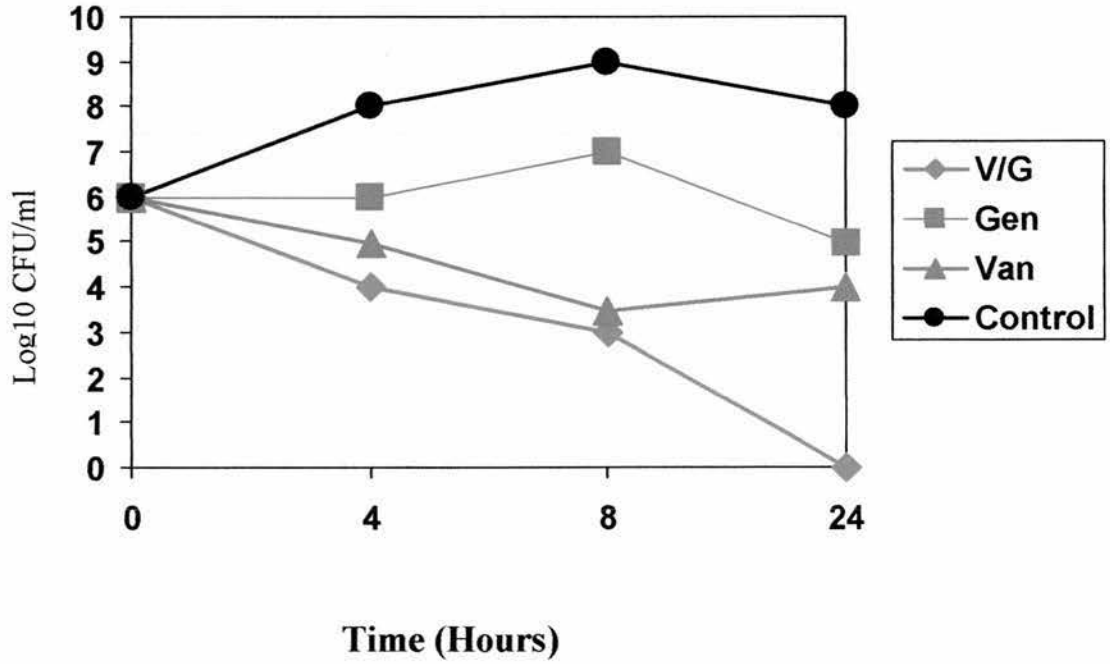


Fig 4.25 Kinetic curves of *E.faecalis* (18B/578) exposed to gentamicin (64mg/l) combined with vancomycin (8mg/l).

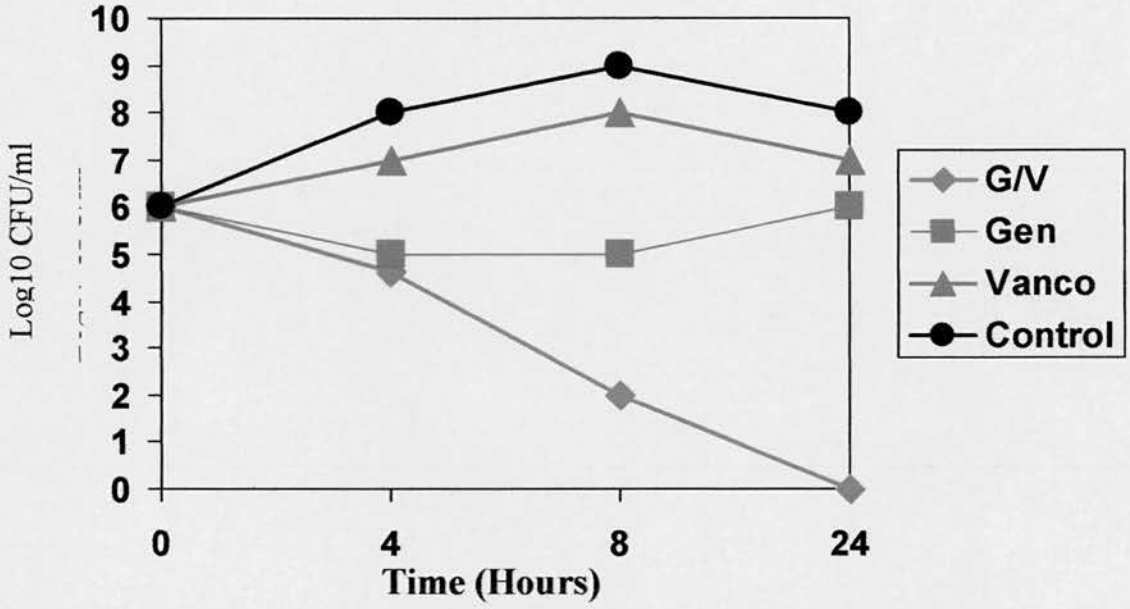


Fig 4.26 Kinetic curves of *E.faecium* (G051) exposed to vancomycin (64mg/l) combined with gentamicin (16mg/l)

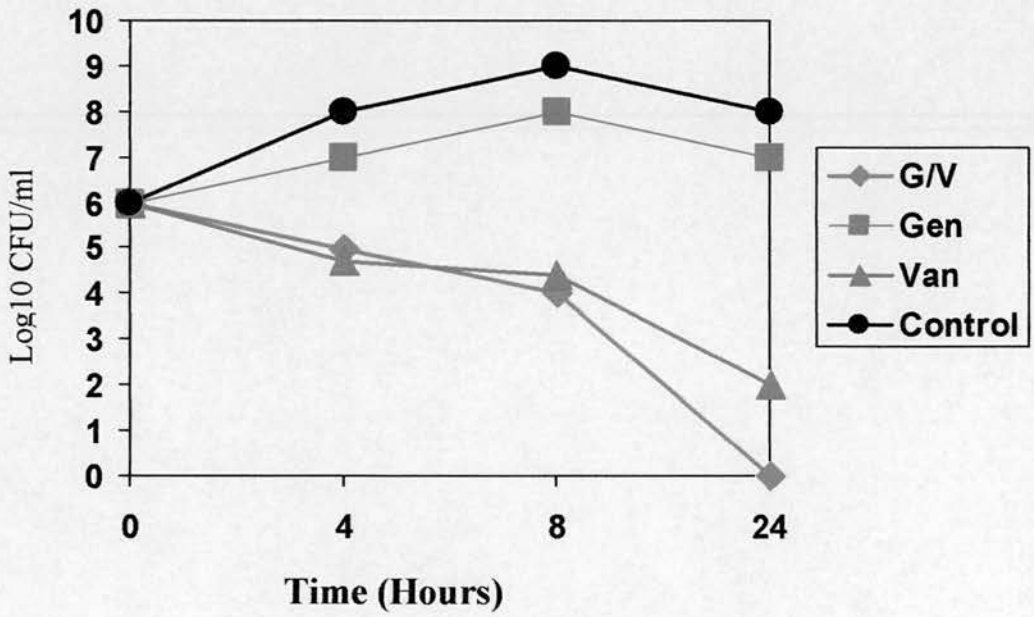


Fig 4.27 Kinetic curves of *E.faecium* (18B/33) xposed to gentamicin (64mg/l) combined with vancomycin (4mg/l). G/V=Combined gentamicin/vancomcin, Gen=gentamicin, Van=vancomycin.

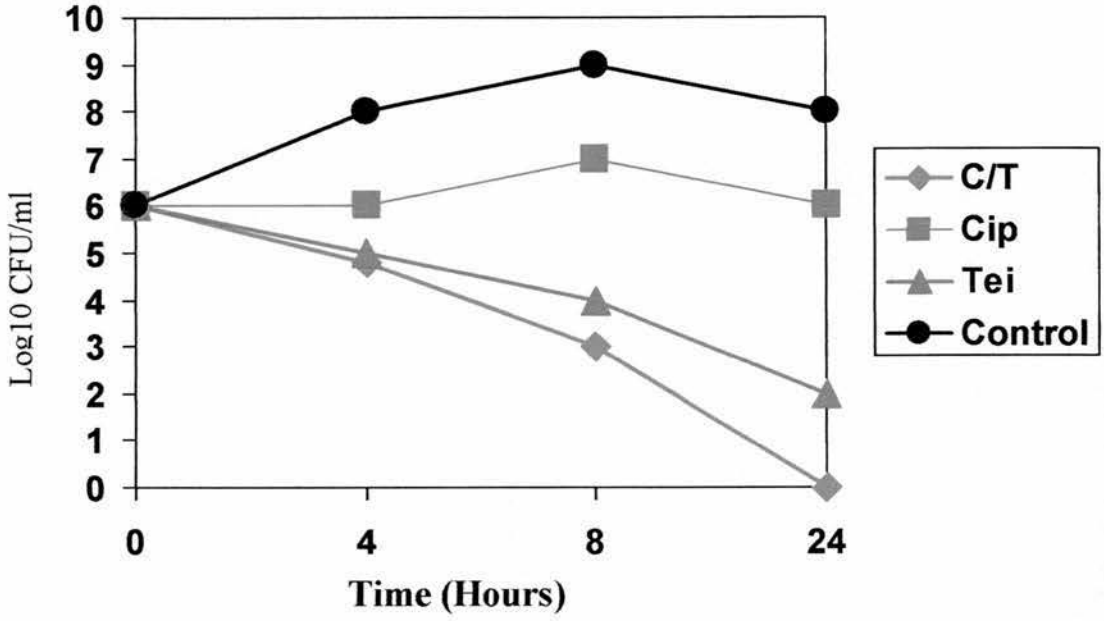


Fig 4.28 Kinetic curves of *E.faecium* (19B/471) exposed to ciprofloxacin (64mg/l) combined with teicoplanin (2mg/l)

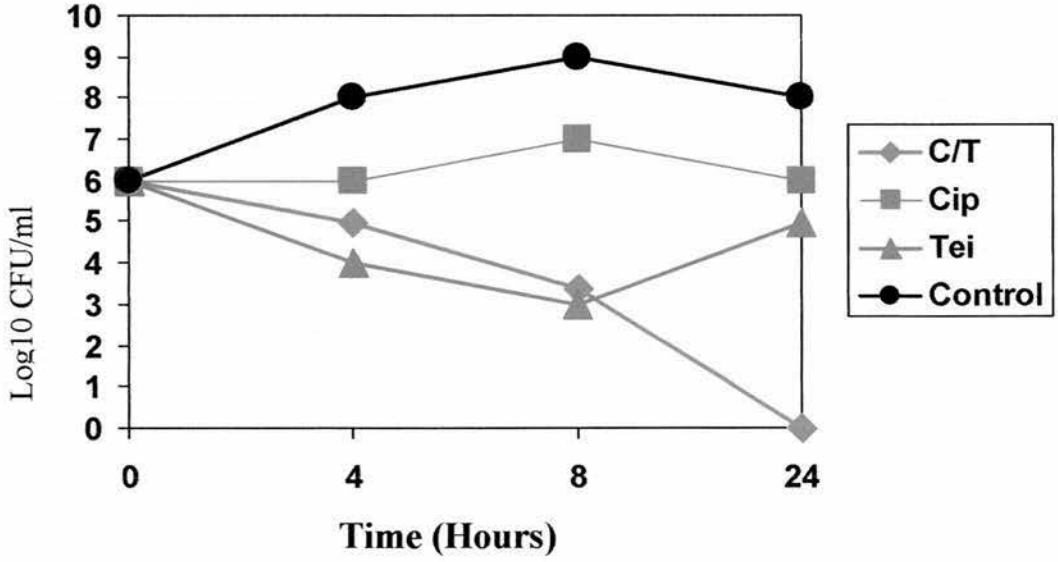


Fig 4.29 Kinetic curves of *E.faecalis* (19B/412) exposed to ciprofloxacin (64mg/l) combined with teicoplanin (2mg/l)

C/T=Combined ciprofloxacin/teicoplanin, Cip=ciprofloxacin, Tei=teicoplanin

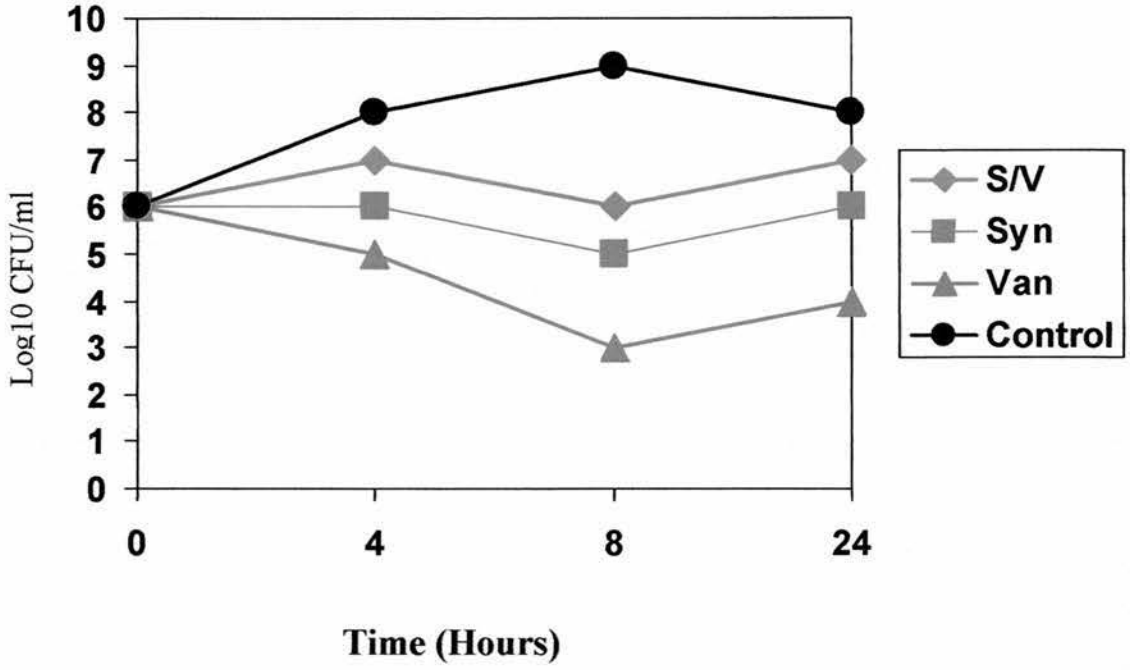


Fig 4.30 Kinetic curves of *E.faecium* (18B/487) exposed to synercid (64mg/l) combined with vancomycin (8mg/

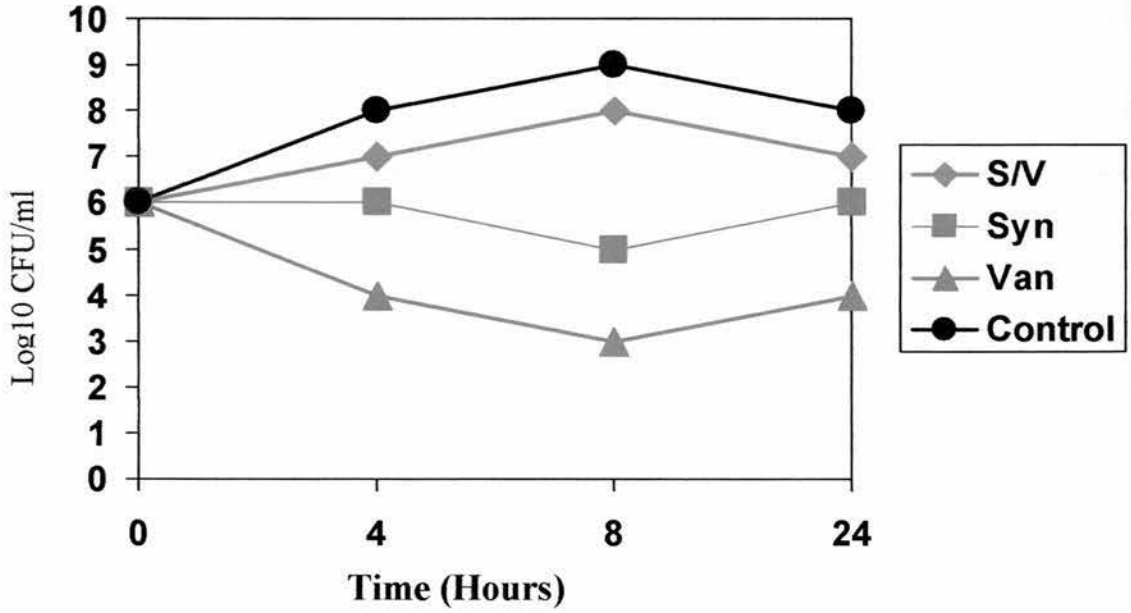


Fig 4.31 Kinetic curves of *E.faecium* (18B/506) exposed to synercid (64mg/l) combined with vancomycin (8mg/l)

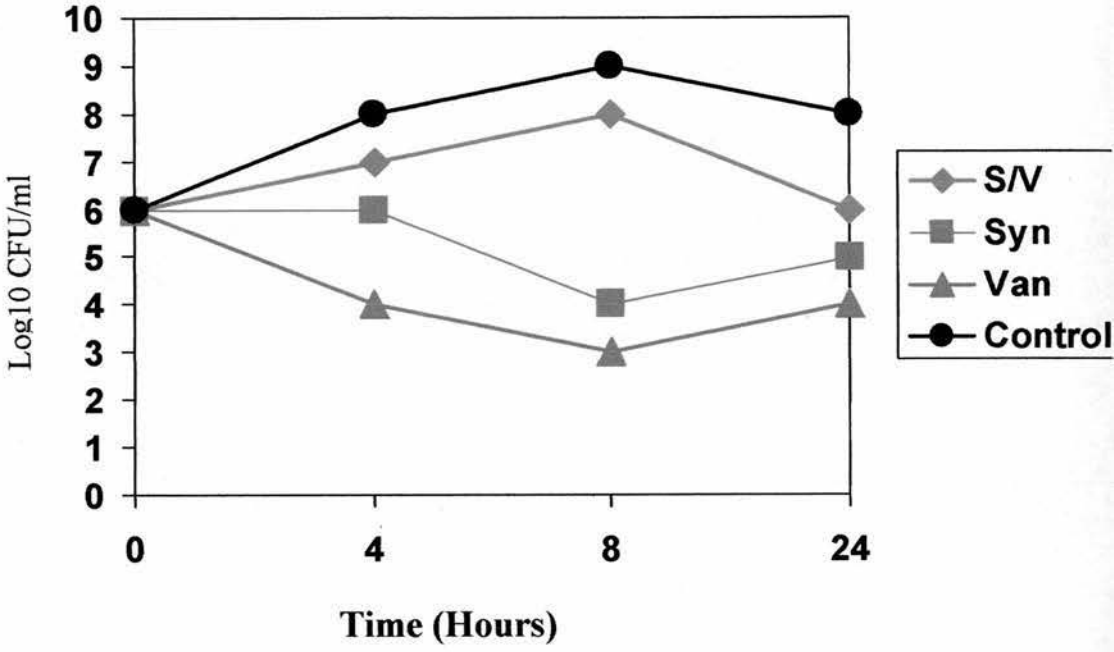


Fig 4.32 Kinetic curve of *E.faecalis* (18B/791) exposed to synercid (16mg/l) combined with vancomycin (4mg/l)

S/V=Combined synercid and vancomycin, Syn=synercid, Van=vancomycin.

CHAPTER 5: Results-Identification of Resistance among the Strains against ciprofloxacin

The purpose of this chapter is to identify, with the aid of PCR technique, the mechanisms of resistance to ciprofloxacin in those strains shown to be resistant, based on the MICs results in section 2.2.3. Ciprofloxacin was the first fluoroquinolone drug widely used in the clinical settings. Originally, it was developed for treatment of infections caused by gram-negative bacteria. Its activity against enterococci appears to be moderate but the resistance against quinolone drugs by enterococci is common among clinical enterococcal isolates. Quinolones inhibit bacteria by interacting with DNA gyrase A and topoisomerase IV, which are required by bacteria for replication. Quinolone resistance has not been as well studied in enterococci as compared to staphylococci and pneumococci. Kanematsu *et al*, in 1998, found an *E.faecalis* isolate with a mutation in *par*C but not the *gyr*A gene with intermediate level of resistance to quinolone but it had higher MIC than an *E.faecalis* with no *par*C or *gyr*A mutations. However, this isolate had lower MIC than *E.faecalis* isolate with mutation in both *par*C and *gyrA*.

5.1 Identification of *gyr*A and *par*C Resistance among Isolates by PCR Method

Resistance to quinolones appears to be due to mainly the alteration in the *gyr*A subunit of DNA gyrase or in the *par*C of topoisomerase IV (Kanematsu *et al*, 1998). It has been demonstrated that quinolones resistance in *E.faecalis* is associated with alteration in *gyr*A (Korten *et al*, 1994). Kanematsu *et al*, 1998, suggested in their findings that the alteration of *gyr*A and *par*C is associated with the development of

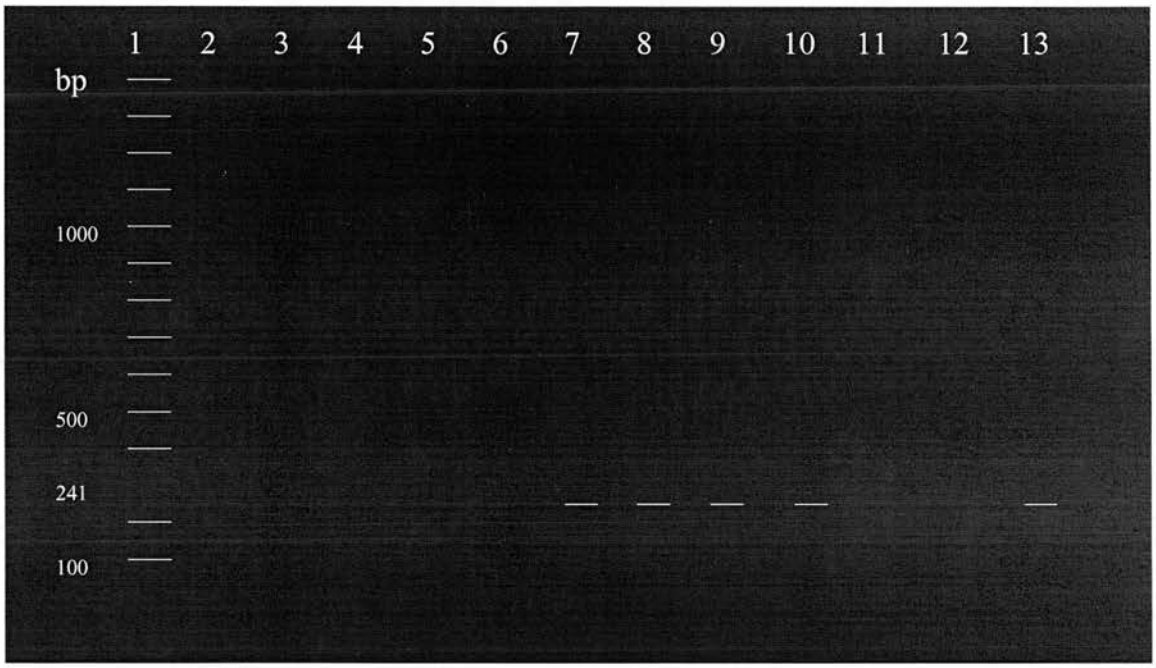
high-level fluoroquinolone resistance in the clinical isolates of *E.faecalis*.

All the *E.faecalis* isolates (including those found with AME resistance gene *–aac(6')-aph(2")* in Section 6.1) were selected for the investigation of the presence of *gyr*A based on MIC >256mg/l for each isolate. Freshly extracted DNA using Helena BioScience extraction kit from each isolate grown overnight in BHI broth at 37°C based on Protocol in Section 2.2.10 was used as template for amplification with a set of primers for *gyr*A (Section 2.2,15). In many occasions, PCR products did not show any fragment when examined in agarose gel electrophoresis with the aid of a computer system. After several attempts, suddenly, there appeared fragments size in one group at 241bp (Fig 5a) when examined in gel electrophoresis. The results showed that 4 isolates (18B/379, 18B/414, 18B/911 and NCTC29212) had fragment size of 241bp in agreement with the predicted fragment of the primers; but one isolate (18B/376) had faint banding at 241bp. The experiment was repeated with the same group to get a good picture of the results in gel. Unfortunately, the saved gel pictures in the computer were lost when the computer got spoiled beyond repair. The repeat of the experiment with freshly extracted DNAas template resulted into observed fragment size of 241bp in agreement with the predicted fragment of primers for 5 isolates when examined in gel electrophoresis (Fig 5.b). The results showed that three isolates (18B/314,18B/390 and 18B/946) which had fragment at 241bp (Fig 5.b) were also found to have AME resistance *aac(6')-aph(2")* in section 6.1. NCTC29212 isolate was used as control. Six *E.faecalis* isolates (3 carrying both *gyr*A and *aac(6')-aph(2")* resistant genes, one carrying only *aac(6')-aph(2")* and two carrying *gyr*A only) were further selected for the investigation of *par*C among the isolates. Freshly extracted DNA from each isolate was used as template and amplification was performed with the primers for *par*C indicated in section 2.2.15. The examination of the PCR products in agarose gel electrophoresis with the aid of a computer system showed

that all the isolates had *par*C type of resistance with fragment at 191bp in agreement with the predicted fragment of primers. The fragments at 191bp for all the isolates were further sequenced for *par*C mutation and the results shown in Table 5.1 and Section 5.2. .

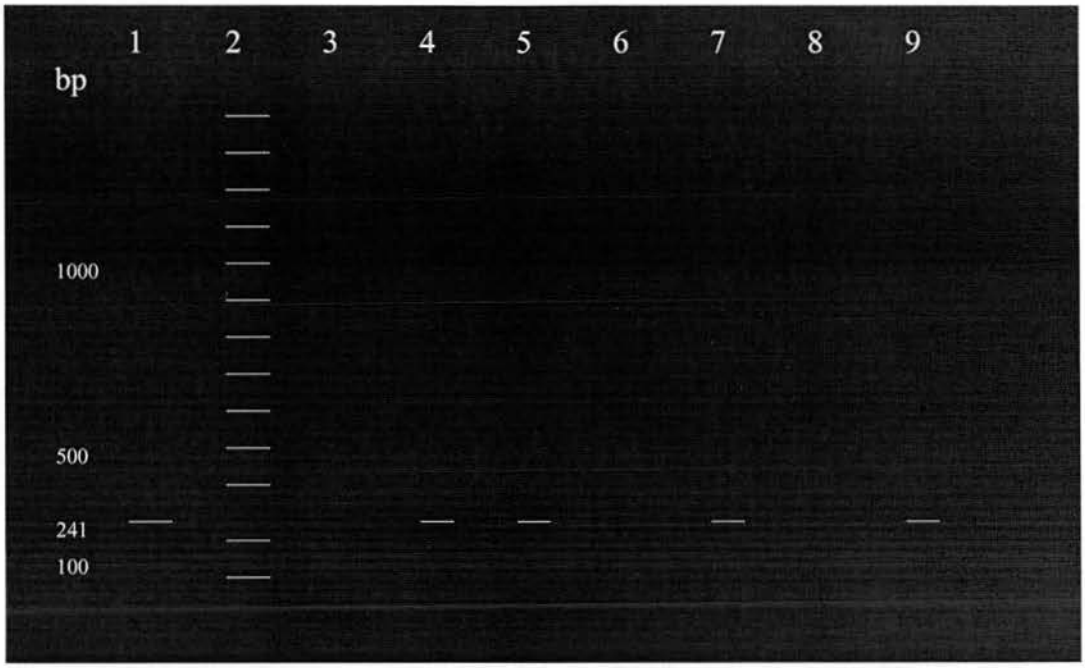
Fig 5.1 (a) & (b) Diagrammatic representation of Gel electrophoresis PCR Products for gyrA

(a)



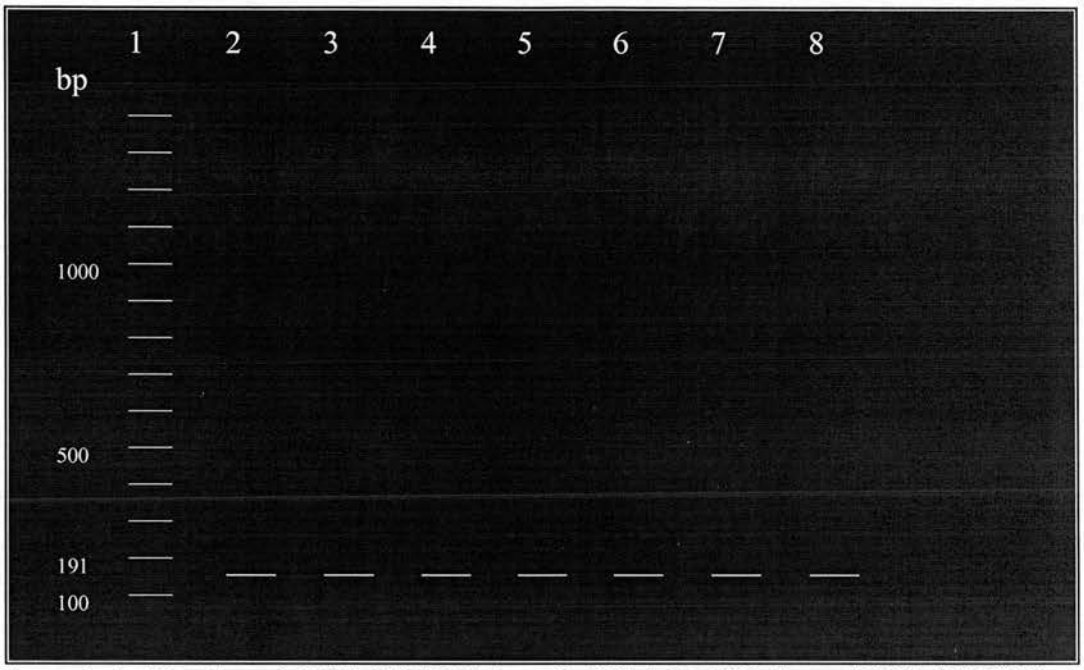
Lane-1-DNA ladder-100bp, lane-2-RIE-17b/407, lane-3-RIE-17B/452, Lane-4-RIE 17B/686, lane-5-RIE-18B/337, lane-6-RIE-18B/358, lane-7-RIE-18B/376, lane-8-RIE-18B/379, lane-9-RIE-18B/414, lane-10-RIE-18B/911, lane-11-RIE-18B/946, lane-12-RIE-18B/976 and lane-13-NCTC29212.

(b)



Lane-1-NCTC29212, lane-2-DNA ladder 100bp, lane-3-RIE-17B/452, lane-4-RIE-18B/390, lane-5-RIE-18B/314, lane-6-RIE-18B/358, lane-7-RIE-18B/946, Lane 8-RIE-18B/976, lane-9-NCTC29212

Fig 5.2 Diagrammatic representation of PCR Products of Gel-electrophoresis for *par*C



Lane 1- ladder; lane 2-RIE-18B/314; lane-3-RIE-18B/376; lane-4-RIE-18B/379; lane-5-RIE-18B/390; lane-6-RIE-18B/414; lane-7-RIE-18B/911 and lane-8-NC TC29212

5.2 Sequencing for parC From PCR products for *E.faecalis*

The sequencing from the products such as PCR allows researchers to identify Mutation(s) that might have occurred in the DNA of a certain species whether bacteria or otherwise . The mutations in the DNA could be lethal or not, depending on the situation. However, the significance of the mutation in the bacterial DNA against a drug is important in that bacteria usually become resistant to such a drug and as a result the treatment of a serious infection caused by such bacteria becomes difficult. The fluoroquinolone resistance in gram-negative organisms is associated with the mutations

in *gyr*A gene (subunit of DNA gyrase) or with a reduced accumulation of the drug. In gram-positive organisms, fluoroquinolone resistance can be associated with mutations in *gyr*A or *par*C (subunit of topoisomerase1V). Although, ciprofloxacin is the drug used frequently in the treatment of infections caused by many gram-negative bacteria, it becomes inactive against the mutated bacteria. In this study, *par*C PCR products of 5 isolates with MICs >256mg/l against ciprofloxacin and one isolate sensitive to ciprofloxacin with MIC of 0.5mg/l (Table 5.2) were sequenced to establish the existence of *par*C mutations in their genes. The results show the existence of changes in their DNA with respect to *par*C. The Overall sequencing appears to be very good (Table 5.1). Isolate RIE-18B/314 with lower MIC of 0.5mg/l had two changes at Ala-80-Val and Ala-85-Ala in its DNA sequence and no change in amino acid. However, Ala subsitituted Ser at position 80 and Val substituted Ile at position 80; whereas RIE-18B/376 had three changes in its DNA sequences at Ser-80-Ile, Ala-85-Ala and Val-87-Val and only one amino acid mutation occurred (Ser-80-Ile). RIE-18B/379 had the change at Ser-80-Ile, Ala-85-Ala and Val-87-Val with one amino acid mutation (Ser-80-Ile) while RIE-18B/390 had three changes at Ser-80-Ile, Ala-85-Ala and Val-87-Val and also only one amino acid mutation (Ser-80-Ile). RIE-18/414 had four changes in its DNA sequences at Ser-80-Ile, Ala-85-Ala, Val-87-Val and Glu-110-Asp with two amino acid mutations (Ser-80-Ile and Glu-110-Asp). RIE-18B/911 had mutation at Ser-80-Ile, Ala-85-Ala and Val-87-Val but only one amino acid mutation occurred (Ser-80-Ile). The partial sequence of *E.faecalis par*C gene obtained from GenBank nucleotide sequence database under the accession no. AB005036 was compared with sequences from test isolates (Table 5.1). **Green** capitals in resistant sequences indicate nucleotides and amino acids different from that of *E.faecalis* AB005036 and also some green capitals

were observed in AB005036 organisms indicating nucleotides and amino acids different from that of test organisms.

Table 5.1 Amino acid changes in *Par*C of *E.faecalis*

RIE-18B/314

```
                101       110       120       130       140       150
                |---------+---------+---------+---------+---------|
3418-parC       GACAGCAGTATTTATGAAGCGATGGTCCGTCTAAGTCAAGACTGGAAATT
AB005036        GACAGTAGTATTTATGAAGCAATGGTCCGTCTAAGTCAAGACTGGAAATT
Consensus       GACAGcAGTATTTATGAAGCaATGGTCCGTCTAAGTCAAGACTGGAAATT

                151       160       170       180       190       200
                |---------+---------+---------+---------+---------|
3418-parC       ACGGGAAGTACTAATTGAAATGCACGGAAACAACGGAAGTATGGATGGCT
AB005036        ACGGGAAGTACTAATTGAAATGCACGGAAACAACGGAAGTATGGATGGCG
Consensus       ACGGGAAGTACTAATTGAAATGCACGGAAACAACGGAAGTATGGATGGCg
```

RIE-18B/376

```
                101       110       120       130       140       150
                |---------+---------+---------+---------+---------|
3522-ParC       GACATTAGTATTTATGAAGCGATGGTGCGTCTAAGTCAAGACTGGAAATT
AB005036        GACAGTAGTATTTATGAAGCAATGGTCCGTCTAAGTCAAGACTGGAAATT
Consensus       GACAgTAGTATTTATGAAGCaATGGTcCGTCTAAGTCAAGACTGGAAATT

                151       160       170       180       190       200
                |---------+---------+---------+---------+---------|
3522-ParC       ACGGGAAGTACTAATTGAAATGCACGGAAACAACGGAAGTATGGATGGCG
AB005036        ACGGGAAGTACTAATTGAAATGCACGGAAACAACGGAAGTATGGATGGCG
Consensus       ACGGGAAGTACTAATTGAAATGCACGGAAACAACGGAAGTATGGATGGCG
```

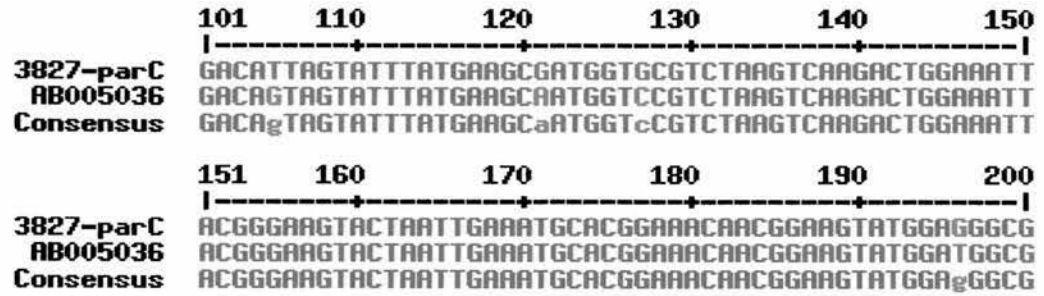
RIE-18B/379

```
                101       110       120       130       140       150
                |---------+---------+---------+---------+---------|
3623-ParC       GACATTAGTATTTATGAAGCGATGGTGCGTCTAAGTCAAGACTGGAAATT
AB005036        GACAGTAGTATTTATGAAGCAATGGTCCGTCTAAGTCAAGACTGGAAATT
Consensus       GACAgTAGTATTTATGAAGCaATGGTcCGTCTAAGTCAAGACTGGAAATT

                151       160       170       180       190       200
                |---------+---------+---------+---------+---------|
3623-ParC       ACGGGAAGTACTAATTGAAATGCACGGAAACAACGGAAGTATGGATGGCG
AB005036        ACGGGAAGTACTAATTGAAATGCACGGAAACAACGGAAGTATGGATGGCG
Consensus       ACGGGAAGTACTAATTGAAATGCACGGAAACAACGGAAGTATGGATGGCG
```

RIE-18B/390

```
                101       110       120       130       140       150
                |---------+---------+---------+---------+---------|
3726-ParC       GACATTAGTATTTATGAAGCGATGGTGCGTCTAAGTNAAGACTGGAAATT
AB005036        GACAGTAGTATTTATGAAGCAATGGTCCGTCTAAGTCAAGACTGGAAATT
Consensus       GACAgTAGTATTTATGAAGCaATGGTcCGTCTAAGTnAAGACTGGAAATT

                151       160       170       180       190       200
                |---------+---------+---------+---------+---------|
3726-ParC       ACGGGAAGT-CTAATTGAAATGCACGGAANCANCGGAAGTATGGATGGCG
AB005036        ACGGGAAGTACTAATTGAAATGCACGGAAACAACGGAAGTATGGATGGCG
Consensus       ACGGGAAGT.CTAATTGAAATGCACGGAAaCAaCGGAAGTATGGATGGCG
```

RIE-18/414



RIE-18B/911

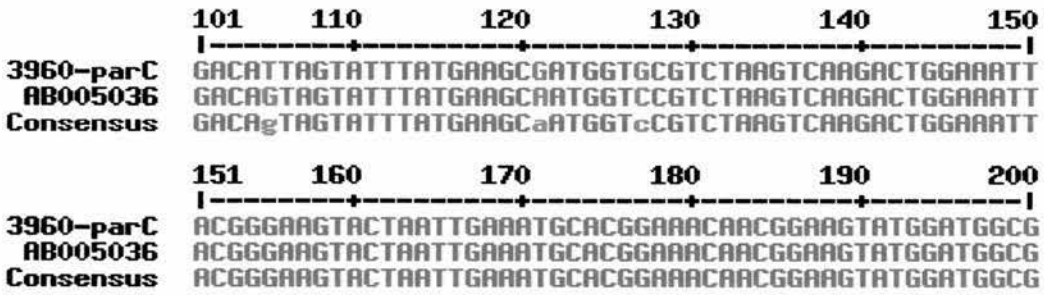


Table 5.2 Susceptibility of *E.faecalis* against Ciprofloxacin

Isolate #	Resistant (MIC)	Sensitive (MIC)
RIE-18B/314	-	S (0.5mg/l)
RIE-18B/376	R (>256mg/l)	-
RIE-18B/379	R (>256mg/l)	-
RIE-18B/390	R (>256mg/l)	-
RIE-18B/414	R (>256mg/l)	-
RIE-18B/911	R (>256mg/l)	-

R= Resistance, S= Sensitive

Chapter 6: Results-Identification of Aminoglycoside-Modifying Enzyme By PCR Method.

This chapter focuses on plasmid or transposon-mediated enzymes involved in the resistance of enterococci (ie *E.faecalis* and *E.faecium*) against an aminoglycoside drug(gentamicin).

6.1 Detection of *aac(6')-aph(2")* enzyme by PCR Method

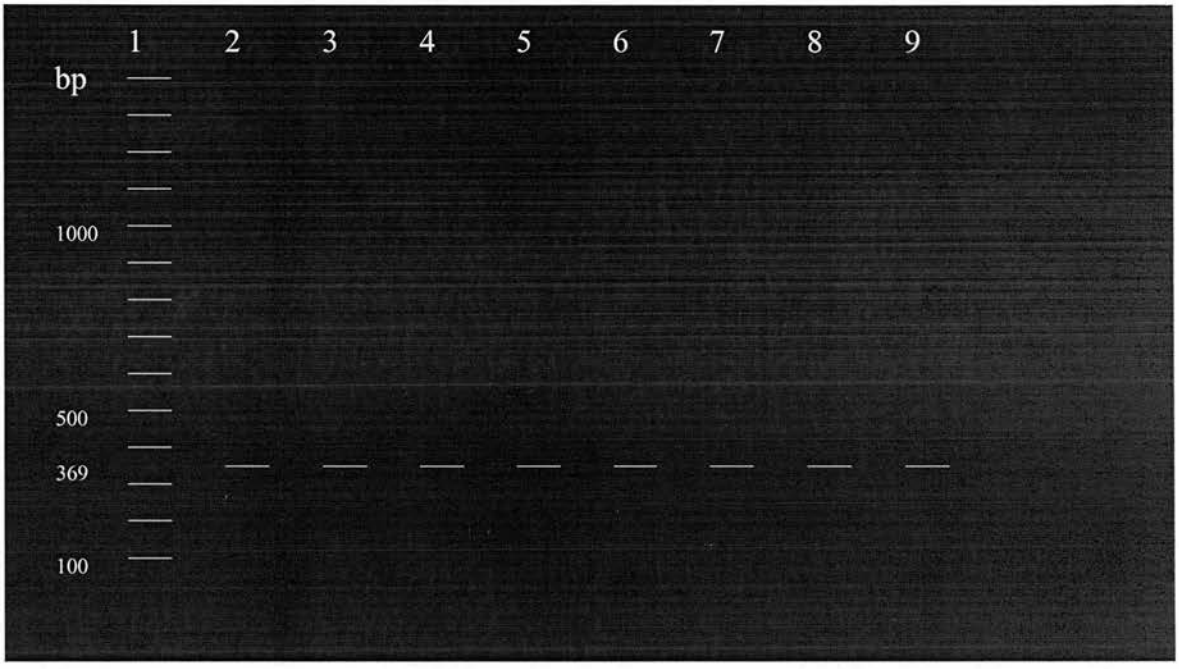
Resistance to aminoglycoside drugs usually involves enzymatic modification of the drugs by aminoglycoside-modifying enzymes (AME) which are found in some plasmids or transposons (Ferretti *et al*, 1986). There are three classes of enzymes involved in the modification of the drugs : acetyltransferases (AACs), adenylyltransferases (ANTs) and phosphotransferases (APHs)(Ferretti *et al*, 1986). The first high-level gentamicin–resistant *E.faecalis* isolate was reported in France in 1979 (Horodneceanu *et al*, 1979). It was confirmed in the subsequent studies that the resistance was due to the fusion of *AAC(6')* with *APH (2")* resulting into bifunctional resistance enzyme with *AAC(6')- APH (2")* activities (Ferretti *et al*, 1986). The gene *aac(6')-le-aph(2")-la* is responsible for encoding a bifunctional enzyme [*AAC(6')-APH(2")*] (Azucena *et al*, 1997; Tsai *et al*, 1998; Kao *et al*, 2000; Chow *et al*, 2001). The gene is generally found on transposable elements such as Tn*5281*-like transposon found in *E.faecalis* (Hodel-Christian and Murray,1991). The studies by Simjee *et al* in 1999 found Tn*5281*-like transposon to be present in *E.faecium* isolate as well. The three other aminoglycoside resistance genes present in some *Enterococcus* species are *aph(2")-lc*, *aph(2")-1b* and *aph(2")-1d* . These genes together with *aac(6')-le-aph(2")-la* gene eliminate the synergistic activities achieved

by combination of gentamicin with the cell- active antimicrobial agents such as ampicillin (Tsai *et al* 1998; Kao *et al*,2000). The mobile nature of these genes, permits intergenus transfer to the other gram-positive bacteria such as staphylococci as well, where it will result in the production of a high-level-gentamicin resistant organism (Thomas and Archer,1989). In order to detect the presence of AME resistance gene *aac(6')-aph(2")* among the isolates, Thirty-two *E.faecalis* and 20 *E.faecium* with MICs >256mg/l each for gentamicin were selected for the investigation with the aid of PCR technique as per section 2.2.7 and Table 2.4. Freshly extracted DNA (using Helena Kits) from each isolate grown overnight on BHI broth at 37°C was used as template during the amplification with the pair of primers having the predicted fragment size of 248bp. However, in each case, the PCR product did not yield any fragment observed in the agarose gel electrophoresis with the aid of a computer system. Attempts were made to optimize the PCR and repeated the experiment but without success. Therefore, another pair of primers (forward-5'-CAGGAATTTATCGAAA ATGGTAGAAAAG-3' and reverse 5'-CACAATCGACTAAAGAGTACCAATC-3') with predicted fragment size of 369bp was used in the amplification of the DNA sequence of the freshly extracted DNA for each isolate grown overnight at 37°C on BHI broth. The examination of the PCR product of the first amplification with the new pair of primers on agarose gel electrophoresis showed some isolates (5 *E.faecalis* and 2 *E.faecium*) with fragment size of 369bp (Fig 6.1b) in agreement with fragment predicted in the primers above. The repeat of the experiment with the isolates which had positive results (Fig 6.1b) and another isolate (18B/900) which had not been amplified before and using the same procedure as above indicated that all the isolates had fragment size at 369bp (Fig 6.1a) when examined in agarose gel electrophoresis and thus in agreement predicted in the primers above. Subsequent amplification of the DNA of the

remaining isolates with the above primers and following the procedure as above yielded 4 positive results (18B/976,18B/946, 18B/911 and 17B/452) (Fig 6.1(c)). Only 10 of 32 *E.faecalis* and 2 of 20 *E.faecium* isolates were found to be harbouring *aac(6')-aph(2")* enzymes which showed high–level gentamicin- resistance among the isolates in this study [Fig 6.1(a), (b) & (c)]. However, Multiplex PCR technique was used for the investigation of other aminoglycoside resistance genes that mediate resistance to gentamicin such as *aph(2")-1b*, *aph(2")-1c* and *aph(2")-1d* among the isolates (32 *E.faecalis* and 20 *E.faecium*) selected on the basis of MICs >256mg/l each. Unlike the standard PCR that uses single PCR primer pairs for each resistance gene in each reaction tube, this technique combines all three primer pairs of each resistance gene in a single PCR tube mixture for single reaction. In this study, 5µl of the freshly extracted DNA of each of the above isolates grown overnight on BHI broth at 37°C was used as template in the PCR mixture. The examination of the PCR products on gel electrophoresis showed no banding with any size of the resistance genes indicated in Table 2.4. Therefore, none of the isolates (32 *E.faecalis* & 20 *E.faecium*) harbours resistance gene shown in Table 2.4

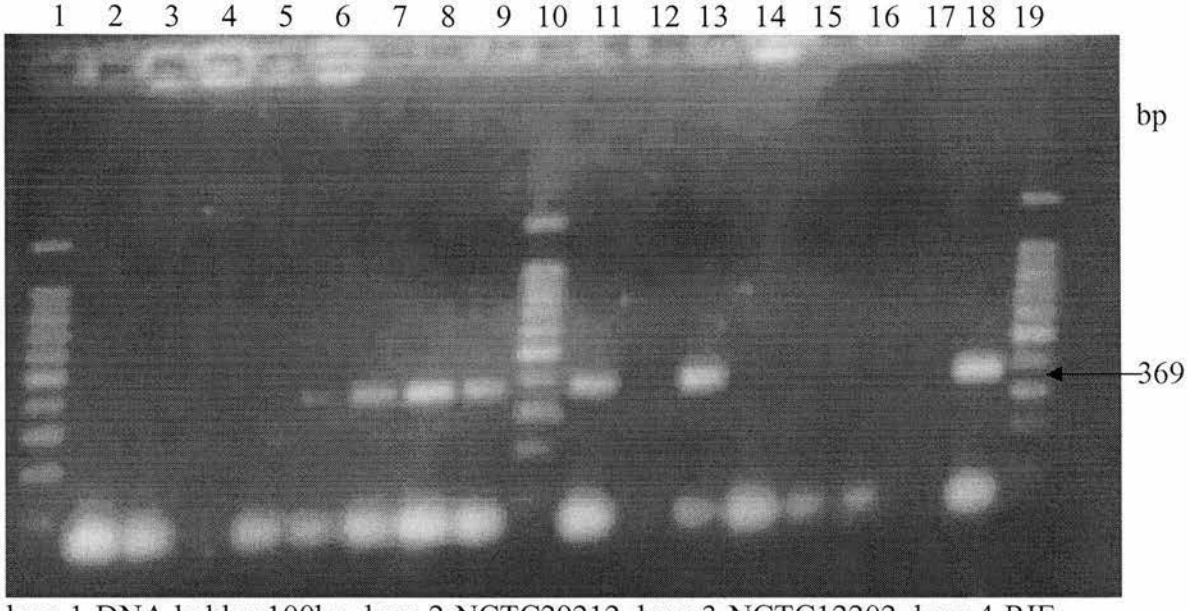
Fig 6.1 (a) Diagrammatic representation of Gel-electrophoresis PCR Products of *AAC(6')-APH(2")*

(a)



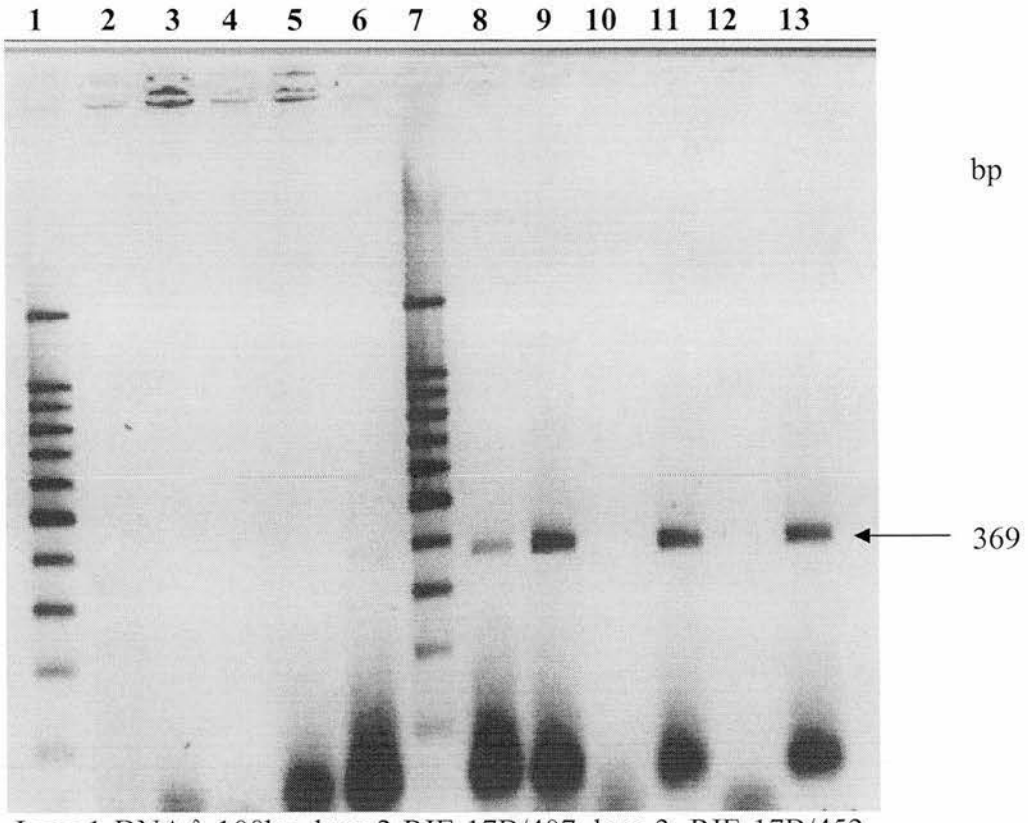
Lane-1-DNA-ladder 100bp, lane-2-RIE-18B/294, lane-3-RIE-18B/298, lane-4-RIE-18B/309, lane-5-RIE-18B/314, lane-6-RIE-18B/329, lane-7-RIE-18B/358, lane-8-RIE-18B/390, lane-9-RIE-18B/900

Fig 6.1 (b)



lane-1-DNA ladder-100bp, lane-2-NCTC29212, lane-3-NCTC12202, lane-4-RIE-18B/234, lane-5-RIE-18B/254, lane-6-RIE-18B/294, lane-7-RIE-18B/298, lane-8-RIE-18B/309, lane-9-RIE-18B/314, lane-10-DNA ladder-100bp, lane-11-RIE-18B/329,lane-12-RIE-18/337, lane-13-RIE-18B/358, lane-14-RIE-18/376, lane-15-RIE-18B/379, lane-16-RIE-18B/382, lane-17-RIE-18B/387, lane-18-18B/390, lane-19-DNA ladder-100bp.

Fig 6.1 (c)



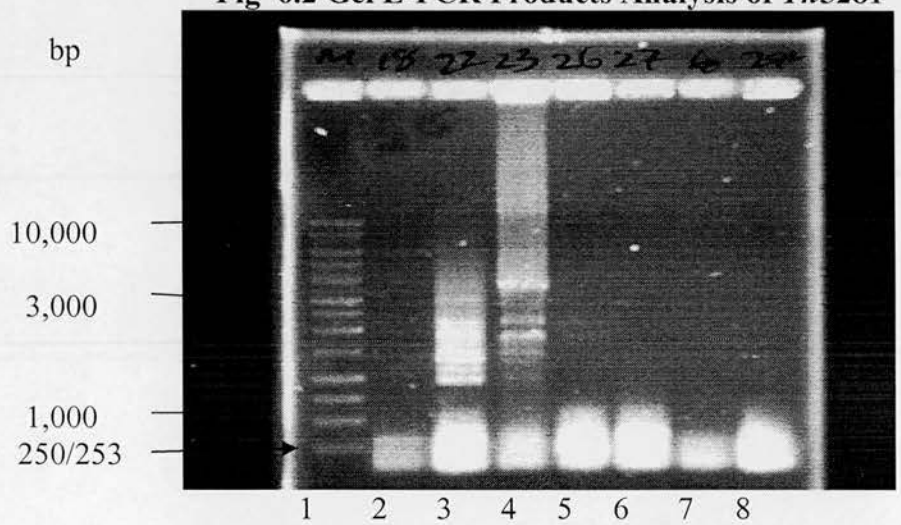
Lane 1-DNA λ 100bp, lane 2-RIE-17B/407, lane 3- RIE-17B/452, lane 4-RIE-17B/686,Lane 5-RIE-17B/849, lane 6-RIE-18B/337, lane 7-DNA λ 100bp, lane 8-RIE-18B/376, Lane 9-RIE-18B/379, lane 10-RIE-18B/387, lane 11-RIE-18B/414, lane12-RIE-18B/487, lane 13-RIE-18B/911,

6.2 Screening of *E.faecalis* for Tn*5281*-Like Transposon by Long-PCR Method

Since Hodel-Christian and Murray in 1991, identified enzymes *aac(6')-aph(2")* as being part of a transposon and designated it as Tn*5281*, Long-PCR protocol was established to identify the presence of Tn*5281* (Simjee *et al,* 2000) and other Transposon such as Tn*4001* (Hodel-Christian and Murray,1990,1991). It is also documented that Tn*5281*-like Transposon which harbours *aac(6')-aph(2")* gene is also flanked by IS*256* sequences in inverse orientation. Therefore any PCR primer used as

forward primer would also anneal to the inverted IS*256* sequence and in this situation, only a single IS*256* primer was required for amplification reaction. In order to screen *E.faecalis* for the presence of Tn*5281*-like Transposon using L-PCR technique, seven *E.faecalis* isolates detected (Section 6.1) to be carrying AME resistant gene- *aac6'-aph2*"were selected for the investigation. Freshly extracted DNA (using Helena BioSciences Kit) each from the seven isolates of *E.faecalis* grown overnight at 37°C in BHI broth were used in the amplification with a single primer (Section 2.8) based on the protocol of ABgene, Epsom, Surrey, UK. The examination of agarose gel electrophoresis for L-PCR product indicated the existence of the bandings for all isolates (Fig 6.2).

Fig 6.2 Gel L-PCR Products Analysis of *Tn*5281



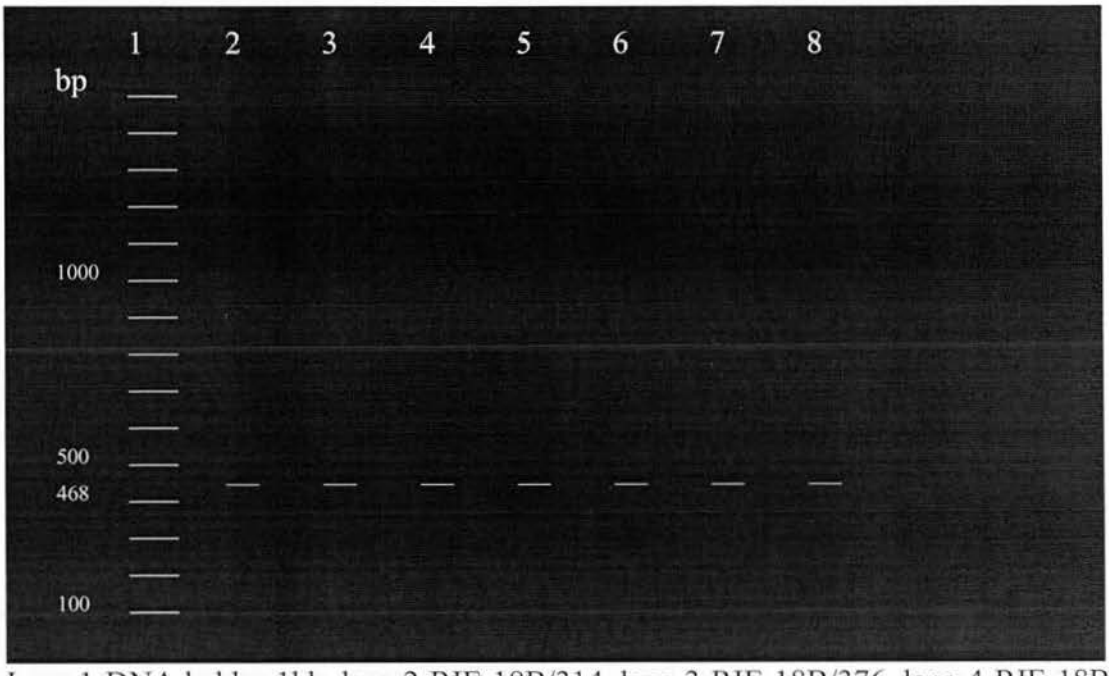
lane-1-DNA ladder-1kb, lane-2-RIE-18B/314, lane-3-RIE-18B/376, lane-4-RIE-18B/379,lane-5-RIE-18B/390, lane-6-RIE-18B/414.Lane-7-RIE-18B/911, lane-8-NCTC29212.

6.3 Detection of IS256 Elements from L-PCR Products with Restriction with *Bam*H1 Enzyme

Lyon *et al* in 1984, described IS*256* as inverted repeats flanking the *aac(6')-aph(2")* bifunctional aminoglycoside modifying enzyme found in *Staphylococcus aureus* which was designated as Tn*4001* transposon. In 1991, Hodel-Chritian and Murray were first to describe the Tn*5281* which was flanked by inverted copies of IS*256*. IS*256* was also found to be involved in the mobility of MLS_B and mercury resistance in addition to aminoglycoside resistance in Tn*5384* (Rice *et al,*1995). The products of L-PCR of the seven *E.faecalis* isolates from Section 6.2 were further studies using restriction endonuclease *Bam*HI described by Dyke *et al,* 1992. The L-PCR Product of each of the seven isolates was incubated with *Bam*HI restriction enzyme for four hours at 37°C.

The examination of the agarose gel electrophoresis of the restricted L-PCR products of the seven isolates stained with Eth.bromide indicated the presence of the fragments at 468bp (Fig 6.3) as predicted in the primer.

Fig 6.3 Diagrammatic representation of Gel-electrophoresis of Restricted L-PCR Products for IS*256*



Lane 1-DNA ladder-1kb, lane 2-RIE-18B/314, lane-3-RIE-18B/376, lane-4-RIE-18B/379, lane-5-RIE-18B/390, lane-6-RIE-18B/414, lane-7-RIE-18B/911 and lane-8-NCTC29212

Chapter 7 : Discussion

Enterococci have emerged in the late part of 20th century as a major cause of nosocomial colonization and infections in Europe as well as in USA. Whilst, within the enterococcal species, *E.faecalis* causes the majority of human enterococcal infections while *E.faecium* accounts for 10% overall. The infections usually include abdominal wound infections, urinary tract infections, bacteraemia and endocarditis. However, the acquisition of resistance to multiple antibiotics including vancomycin, penicillin and aminoglycosides by enterococci has made these bacteria a major health problem and also contributes to their pathogenicity within the hospital settings. On the other hand, the use of antibiotics such as vancomycin and avoparcin in animal feed for growth promotion and infectious disease prophylaxis (Witte, 1998) may also contribute to prevalence of some multidrug-resistant organisms harbouring plasmids and transposons through the food chain from the animal products although no evidence has been established. However, there has been controversy with respect to the use of avoparcin in animal feed which led to its withdrawal for use in livestock animal production within the animal husbandry and pharmaceutical industries in European union. The same kind of five-gene *van*RSHAX resistance to avoparcin occurs in animals and can be transmitted zoonotically (Witte, 1998). Many studies are addressing the level of relatedness of *van*A GRE of human and non-human areas. Since 1988, a rapid rise in the incidence of infections and colonizations with vancomycin-resistant enterococci (VRE) has been reported by

National Nosocomial infections survellance system (NNIS), 1999. This is a cause for concern since there is a lack of availability of antimicrobial therapy for the treatment of VRE infections and most VRE strains harbour resistance to multiple antibiotics such as gentamicin, ampicillin and vancomycin which are often regarded as antibiotics of the last resort. The major concern is the transfer of vancomycin resistance genes fromVRE to other gram-positive bacteria such as *Staphylococcus aureus* (i.e now called vancomycin resistant *S.aureus*) which is a serious public health concern (MMWR, 2000) because there are no good treatments for *S.aureus* strains with both MRSA and VRSA phenotypes. Enterococci are relatively resistant to β-lactams. *E.faecium* is inherently more resistant to penicillin than *E.faecalis* (Moellering *et al*, 1979; Gordon *et al*, 1992). However, the resistance to multiple antibiotics by enterococci has prompted the search for newer or combinations of antibiotics which may result into discovery of the most powerful antibiotic(s) to aliviate the problem of infections caused by the resistance organisms. The application of technique such as PFGE reveals heterogeneous population of bacteria within the hospitals unless the strains are associated with a defined outbreak. Bacterial diversity is thus at the root of any significant resistance mechanism. The diversity of HLGRE and GRE isolates points to the disseminations of AME {e.g *aac(6')-le-aph(2")-la, aph(2")-lb*, *aph(2")-lc* and *aph(2")-ld* } and *Van*A types of resistance respectively through transfer of plasmids and transposons. The investigations have turned into looking at the diversity of Tn*5281* elements for AME and Tn*1546* elements for *Van*A respectively. It has also been documented that the combination of aminoglycoside with gylcopeptide or β-lactam resulting in the synergistic activity has helped to aliviate some problems of endocarditis although, high-level resistance to gentamicin has emerged in USA creating therapeutic problems for patients with serious infections such endocarditis which is usually treated with a cell-wall-active agent

combined with aminoglycoside (Murray, 1990). In this study, 52 *E.faecalis* and 21 *E.faecium* isolates of gentamicin resistance were collected from RIE to gain some understanding of the combinations of antibiotics with aim of establishing synergistic activity based on the techniques such as Agar dilution, Time-kill curves and Checkerboard. In addition, isolates were examined with respect to acquisition of AME resistance genes such as *aac(6')-le-aph(2")-la*, *aph(2")-lb*, *aph(2")-lc* and *aph(2")-ld* and also the presence of *gyr*A and *par*C mutations among the isolates against ciprofloxacin as well as PFGE technique for diversity among the isolates .

7.1 The composition of Gentamicin-resistant Isolates Studied

The composition of the gentamicin–resistant clinical isolates from RIE consists of 52 *E.faecalis* and 21 *E.faecium*. Since Horodniceanu *et al* reported in 1979 the *E.faecalis* isolates with resistance to gentamicin with MIC ≥ 16.000mg/l, high-level resistance to gentamicin involving both *E.faecalis* and *E.faecium*, has now been shown to be endemic to many regions of the USA and countries outside USA including Thailand, Japan, Italy, Chile , Greece and UK (Hoffman *et al*, 1987; Bendall *et al*, 1991; Woodford *et al* 1993; Paparaskevas *et al*, 2000). Most of the enterococcal infections such as endocarditis, urinary tract infections, bacteraemia and intra-abdominal infections are caused by multi-drug resistant enterococci. *E.faecalis* is the most predominant in enterococcal infections (80%) and 10% being *E.faecium* Perhaps, this is an indication that *E.faecalis* is more virulent than *E.faecium*. In most cases, the collection of enterococcal isolates gathered to show gentamicin resistance which reflects the prevalence of either *E.faecalis* or *E.faecium* or both. For example, a total of 158 enterococcal isolated from AHEPA University Hospital in Greece during 1993-1994 had high-level gentamicin resistance with MIC >512mg/l

(Tsakris *et al*, 2001). 45.2% were found to be *E.faecalis* and 31.2% were found to be *E.faecium*. Similarly in a tertiary-care hospital in Athens, Greece, where a total of 55 *E.faecalis* and 21 *E.faecium* were obtained, 22% of *E.faecalis* had high-level gentamicin resistance (MIC > 500) but none was found in *E.faecium* (Paparaskevas *et al*, 2000). However, the rate of enterococci isolates from teaching, associated teaching and district general hospital in UK in two consecutive years (Oct,1996 – Jan,1997 and Oct,1997 to Jan,1998), showed increase in high-level resistance to gentamicin by *E.faecalis* with an increase from 10.5% year 1 to 15.1% year 2, but an increase in high-level resistance to gentamicin for *E.faecium* was not significant (ie 12.8% year 1 to 22.5% year 2) (Andrews *et al,* 2000). In this study, 67% of gentamicin resistance organisms (MIC range 32->256mg/l) were *E.faecalis* ; while 32% were *E.faecium* (MIC range 128->256mg/l). This agreed with other findings that the majority of gentamicin-resistant isolates are predominantly *E.faecalis* . The predominance of *E.faecalis* in enterococcal infection reflects the fact that *E.faecalis* may be efficient at acquisition of the resistant genes which are more virulent than *E.faecium*. However, the result of the MIC range of 128->256mg/l of this study may reflect the fact that *E.faecium* are more intrinsically resistant than *E.faecalis*. All the isolates in this study were speciated with the aid of API 20 Strep tests. They were identified as either *E.faecalis* or *E.faecium* or *E.durans* with the indication of strong certainty of the API profile showing excellent to good scores. Ling *et al,* 2002, had similar results when they used API 20 Strep test in their study with the scores of API profile showing an excellent to good in the identification of their enterococcal isolates they collected. In a similar study, Ling *et al*, 2002, also identified 286 isolates (ie 239 *E.faecalis*, 45 *E.faecium*, one *E.gallinarum* and one *E.cassiflavus*) using both API 20 Strep and PCR techniques.

However, commercially-available kits including API 20 Strep system appear not to be reliable in the identification of enterococcal species except for *E.faecalis* (Miranda *et al*, 1991; Morrison *et al*, 1997; Ling *et al,* 2002). Although PCR could correctly identify clinically *E.faecalis* and *E.faecium* as in the case of Ling *et al*, 2002, in this study, the PCR could not reveal the difference between *E.faecium* and *E.durans,* which was identified by API 20 Strep method. Again, PCR method might not be suitable for use in routine clinical laboratories because of the special techniques and equipment required, and also not cost effective if only few isolates are tested at one time. However, under certain circumstances such as testing for the presence of VRE, high-level gentamicin resistance or β-lactamase producing enterococci, PCR could be used. As expected, cultural characteristics of some strains of enterococci on horse blood agar may show alpha-haemolysis or beta-haemolysis (Ananthanarayan and Paniker, 1996). In this study, all *E.faecium* showed α-haemolysis; while *E.faecalis* had or showed a mixture of α- and β-haemolytic characteristics on horse blood agar. The isolation of the coexistence of high-level resistance to aminoglycosides and beta-lactamase production in rare strains of enterococci, particularly *E.faecalis* has been reported in USA in 1981 (Murray and Mederski-Samoraj, 1983) and subsequently in Argentina and Lebanon. These strains had also high-level resistance to gentamicin . The spread of these strains is, of course, worrisome because the spread of both high-level gentamicin resistance and β-lactamase may be responsible for the elimination of synergy of the combined drugs for the treatment of serious infections by enterococci such as *E.faecalis* and *E.faecium*. In this study, based on the MICs of the β-lactam drugs used, it was necessary to establish the presence or absence of the β-lactamase producing isolates in the isolates collected for the study. However, it turned out that none of the isolates was found to be a β-lactamase producer. The isolates used for this study were also collected

on the basis of gentamicin resistance and are more likely to be multi-drug resistant than the enterococcal collection which are not specifically gentamicin resistance. It is therefore essential to establish antibiotic susceptibilities of the collected isolates. Lavery *et al,* 1997, found in their retrospective study of enterococcal isolates that 2% were vancomycin-resistant but found that high-level gentamicin resistance was in the increase from 17% to 60% and that of ampicillin resistance from 22% to 51%, none was found to be a β-lactamase producer. This study also found that none of the isolates was a β-lactamase producer but had high-level of gentamicin resistance as mentioned earlier. In this study, 69% of *E.faecalis* and 66% of *E.faecium* collected from RIE were found to be resistant to ciprofloxacin (MIC>256mg/l). It is assumed that the spread of the resistance was due to clonal spread in RIE although no any study was done to establish the fact. This findings agreed with the the findings of Tankovic *et al*, 1996 which established the increase of resistance of ciprofloxacin from 0% of *E.faecalis* isolate in 1986 to 24% in 1992 reflecting the clonal spread of resistant isolates. Since 69% of *E.faecalis* and 66% of *E.faecium* were found to be resistant to ciprofloxacin in this study, the newer quinolone drug- moxifloxacin, had more improved activity against isolates (*E.faecalis* and *E.faecium*) with only 28% resistance in the same study. This agreed with the previous findings by Hoogkamp-Korstanje and Rollofs-Willeme, 2000. Both vancomycin and teicoplanin are drugs belonging to glycopeptides group which share similar antibacterial spectra and potencies which are confined to gram-postive bacteria. Their activities are not identical. Whereas, vancomycin binds to the growing cell wall by dimerization thus enhances its activity against bacterial cells, teicoplanin, on the other hand, has fatty acid chain which acts as a membrane anchor and increases affinity for the growing cell wall of the bacteria. In this study, teicoplanin was found to be more active against *E.faecalis* (MIC=2mg/l and *E.faecium* (MIC =1mg/l) while vancomycin was

found to be relatively sensitive to both isolates (MIC=8mg/l) in agreement with the previous findings by Cercenado *et al*, 2001. Linezolid is most active drug against gram-positive bacteria including enterococci. All the isolates (*E.faecalis* and *E.faecium*) in this study were found to be sensitive to linezolid in agreement with the previous findings by Eliopoulos *et al*, 2002. Piperacillin is one of the ureidopenicillins active against *P.aeruginosa*. However, it can be used against *E.faecalis* infections. This study has found that piperacillin was relatively active against *E.faecalis* (MIC = 8mg/l) but more resistant against *E.faecium* (MIC =256mg/l). The resistance against *E.faecium* appears to be the intrinsicness of *E.faecium* against β-lactams since none of the isolates was found to be β-lactamase producer. Combination of piperacillin/tazobactam (Tazocin) may have some advantage over co-amoxiclav, ticarcillin/clavulanin or ampicillin/sulbactam because piperacillin is easier to protect against TEM *beta*-lactamases as a result of lower affinity of piperacillin for these enzymes (Livermore, 1993). β-lactamase producing *E.faecalis* strains are usually susceptible to tazocin but *E.faecium* strains with high intrinsic resistance to penicillin G are resistant to this combination (Chen *et al*, 1993 ; Okhuysen *et al*, 1993). Since this study did not detect any β-lactamase producer among the isolates of *E.faecalis* & *E.faecium*, it was found that *E.faecium* was more resistant to the combination (tazocin) (MIC =256mg/l) than *E.faecalis* (MIC=16mg/l). The characteristics of *E.faecium* isolates being more resistant to β-lactam drugs than *E.faecalis* appear to reflect the intrinsic resistance of *E.faecium* to the drugs. *Beta*-lactamase producing strains of *E.faecalis* are sensitive to co-amoxiclav (also known as augmentin) (Ingerman *et al*, 1987). Augmentin was found to be relatively active against *E.faecalis* (MIC=16mg/l) by this study while *E.faecium* was found to be relatively resistant to the combination (MIC=32mg/l). Amoxicillin also known as alpha-amino-p-hydroxybenzyl-penicillin is twice as active as ampicillin against *E.faecalis* (Neu, 1974).

My findings in this study shows that amoxicillin was relatively active against *E.faecalis* (MIC=16mg/l) while *E.faecium* was relatively resistance (MIC=32mg/l) to the combination in agreement with Neu. The combination of quinupristin and dalfopristin (Q/D) which is also called synercid is used in the treatment of infections caused by multidrug-resistant *E.faecium*. Virtually all *E.faecalis* isolates are intrinsically resistant to synercid (Chang *et al*, 1999). However, resistance to synercid among *E.faecium* isolates can be due to the presence of a single gene *vat*D (Rende-Fournier *et al*, 1993). This study found that all *E.faecalis* isolates were resistance to synercid (MIC = 64mg/l) while *E.faecium* was found to be relatively resistant to synercid (MIC=32mg/l).

7.2 Antimicrobial Combinations and Synergy by the *in vitro* Agar method

Although resistance to antimicrobial agents may be due to drug-inactivating enzymes (Benveniste and Davies, 1973) or an insensitive target site (Zimmerman *et al*, 1971) or acquisition of resistance genes, it may also be due to permeability barrier. In such situations, a given drug could be active if another agent alters the permeability of the bacterial cell in order to permit entery. It has been postulated that agents that act on the cell-wall may enhance the entry of aminoglycosides in this manner in a number of bacterial species including *E.faecalis* and *E.faecium* (Moellering & Weinberg, 1971). The original observation of penicillin/aminoglycoside synergism was probably made in 1947 (Hunter, 1947) and subsequently many other investigators have demonstrated a synergistic effect of penicillin plus aminoglycosides against enterococci (Moellering *et al*, 1971). In this study, the combination of amoxicillin and gentamicin against both *E.faecalis* and *E.faecium* shows the production of synergism (MIC=0.5mg/l for *E.faecalis* & MIC=2mg/l for *E.faecium*). It is assumed that synergistic activity between amoxicillin and gentamicin occurred due to amoxicillin active on bacterial cell-wall and

facilitated the entry of gentamicin to act on ribosome 30s subunit thus killing bacteria. Similarly, when vancomycin-active cell-wall drug- was combined with gentamicin against *E.faecalis* and *E.faecium* isolates in this study, synergistic activity occurred (MIC=2mg/l for *E.faecalis* and MIC=1mg/l for *E.faecium*). This agreed with the findings by Moellering & Weinberg, 1971. Such results may indicate that the combinations of amoxicillin/gentamicin and vancomycin/gentamicin may be suitable for treatment of infections caused by both isolates. The combination of teicoplanin active cell-wall drug with synercid against *E.faecalis* and *E.faecium* isolates in this study produced synergistic activity. The combination of teicoplanin/synercid was more effective against both *E.faecalis* and *E.faecium* (MIC=0.5mg/l). The results agreed with the findings of Hill *et al*, 1997 which established synergistic activity of the combination of the two drugs (MIC=0.25mg/l). The results indicate that combination may be useful for treatment of infections caused by the strains of both isolates. When teicoplanin was combined with ciprofloxacin against both isolates (*E.faecalis* & *E.faecium*) in this study, production of synergy occurred. The combination of the two drugs (teicoplanin/ciprofloxacin) was found to be most effective against all isolates (MIC=<0.25mg/l) than all other combinations in this study. The results suggest that the combination may be useful in treatment of serious infections caused by *E.faecalis* and *E.faecium*. Combination of amoxicillin and synercid against *E.faecalis* and *E.faecium* isolates in this study produced synergistic activity (MIC=4mg/l). It is assumed that synergy occurred because amoxicillin being active on cell-wall of bacteria facilitated the entry of synercid into cell and inhibited bacterial protein synthesis by binding to 50S ribosomal subunit and killing bacteria. It is also interesting to note that *E.faecalis* isolates are intrinsically resistant to synercid alone but the combination (amoxicillin/synercid) had made isolates sensitive to the combined drugs. This combination could be useful for treatment of infections caused

by both isolates. On the other hand, the combination of vancomycin and synercid did not produce any synergistic activity against the isolates in this study (MIC=32mg/l for *E.faecalis* and MIC=16mg/l for *E.faecium*). This indicates the antagonistic activity of the combination against the isolates. The findings agreed with the previous findings by Hill *et al*, 1997. Although it is assumed that vancomycin (active cell-wall) might have permitted the entry of synercid to bind 50S of ribosomal subunit of bacterial cell and since synercid is bacteriostatic, it appears, its activity could not withstand the level of bacterial resistance against it. Such combination is not good for treatment of any infection caused by any isolate. The combination of synercid and ciprofloxacin in this study resulted in synergistic activity against *E.faecium* (MIC=4mg/l). However, it is interesting to note that the combination of synercid and ciprofloxacin against *E.faecalis* had reduced the MIC of ciprofloxacin from >256mg/l to 64mg/l and also the fact that *E.faecalis* is intrinsically resistant to synercid shows that activity of the combined drugs was effective against isolates and resulted in bactericidal effect by more than 2 $\log_{10}$ CFU unit indicating synergy.

7.3. Time-kill curves Test for evaluating synergy/antagonism

Drug–resistant enterococci present a major therapeutic problem especially in immunosuppressed patients (NCCLS, 1997; Rice& Shlaes, 1995). Since the development of a new drug for use in clinical therapy takes a long time (ie approximately six years), the other alternative may be to use the combinations of drugs currently being used alone for the treatment of infections caused by the drug-resistant enterococci such as *E.faecalis* & *E.faecium*. One of the techniques used in the evaluation of drug combinations is the time-kill curves. This technique measures the bactericidal rate, duration of the bactericidal effect and if the bacterial regrowth eventually occurred. It has been

documented that time-kill curves studies more aptly reflect the clinical outcome. The present study was performed with an inoculum size of 10^5-10^7 CFU/ml, mimicking clinical conditions (Bingen *et al*, 1990) and colonies were counted after 24hour of incubation. Inoculum size and bacterial growth phase did not affect the assessment of synergy/antagonism at 8 hour with *E.faecalis* and *E.faecium* isolates. The combinations were most efficient at 8 hour with 2 $\log_{10}$ unit decreases in viable counts and showing synergy observed in the study. The activity of the combined amoxicillin and gentamicin against *E.faecalis* and *E.faecium* in this study had resulted into synergy as observed at 8 hour (Figs 4.9- 4.13) while a similar result with the same combination was also observed against the isolates in agar dilution technique in this study. The results show that it may be possible to use the combined drugs for the treatment of infections such as endocarditis caused by *E.faecalis* or *E.faecium*. The synergistic activity was also observed with the combination of teicoplanin and synercid at 8 hour of antimicrobial exposure against *E.faecalis* and *E.faecium* isolates (Figs 4.14 & 4.15) in agreement with the similar activity shown in section 4.2 and previously reported by Hill *et al*, 1997. While bactericidal effect of the combined amoxicillin and synercid against *E.faecalis* and *E.faecium* isolates resulted in synergistic activity (Figs 4.16-4.19), a similar activity by same combination was observed in section 4.2. The results may indicate that the synergy between combinations may likely occur *in vivo* situation as well. Exposure of *E.faecalis* and *E.faecium* isolates against the combined ciprofloxacin and synercid resulted in synergistic activity (Figs 4.20-4.23) and was similar to bactericidal activity of the same combination observed in section 4.2. The combination of ciprofloxacin/synercid may be useful clinically. Combination of teicoplanin and ciprofloxacin against *E.faecalis* and *E.faecium* isolates resulted in synergistic activity (Figs 4.28 & 4.29) and regrowth in teicoplanin. This agreed with the similar activity of the same combination in section 7.2.

Combining vancomycin with gentamicin against *E.faecalis* and *E.faecium* resulted into synergistic activity (Figs 4.24- 4.27) in agreement with the previous findings by Moellering and Weinberg, 1971 and also same activity of the combination observed in section 4.2. On the other hand, combination of synercid with vancomycin against *E.faecalis* and *E.faecium* isolates resulted into antagonistic activity (Figs 4.30-4.32) in agreement with the findings by Hill *et al*, 1997) and a similar activity was also observed in section 4.2.

7.4 Synergy Testing of the Combined Antimicrobial Agents by Checkerboard Method

Patients with infections which are life-threatening or resistant to treatment with single antibiotics tend to do better when treated with antibiotic combinations shown *in vitro* to act synergistically against the pathogenic strains (Beerenbaum, 1978). One of the techniques used in testing the combinations of antibiotics is Checkerboard. This technique has been used most frequently to assess antimicrobial combinations *in vitro* with respect to synergistic therapy in the treatment of neutropenic patients with gram-negativ septicaemia (Klastersky *et al*, 1972; Lau *et al*, 1977). The results of checkerboard test is interpreted in terms of fractional inhibition concentrations (FIC) index with the values of 0.5 for synergy, 1 for additive and 2 for antagonism (Beerenbaum, 1978). The FIC index method allows a more objective analysis of the results than mere drawing of isobologram lines (Holm, 1986). In this study, owing to elaborate nature of setting up each checkerboard agar dilution method to assess the interaction of the combined antimicrobial agents, only four strains of vancomycin resistant *E.faecium* were tested against the combined ciprofloxacin/synercid. Synergistic activities were observed against the strains tested (Figs 4.3-4.6). Two of the four strains (D002 and G051) were further tested against other antimicrobial combinations of gentamicin/vancomycin for G051 and

teicoplanin/synercid for D002 and synergistic activity of the combined drugs was observed (Fig 4.26 for G051 and appendix B for D002). Although synergistic activity of the combined drugs against the two isolates (G051 & D002) was observed, the *in vivo* test should be done to establish clinical usefulness of the combined drugs.

7.5 Detection of Aminoglycoside Modifying Enzymes in Aminoglycoside Resistant enterococci.

Aminoglycoside modifying enzymes (AMEs) are major factors which confer aminoglycoside resistance on bacteria. Since its emergence in 1979, enterococci with high-level gentamicin resistance (HLGR) have been disseminated in most countries and its prevalence was remarkably increased in 1990s (Simjee and Gill, 1997). HLGR is recognized as a clinical serious problem and routine examination including surveillance of clinical isolates of enterococci are necessary for the choice of appropriate treatment and infection control. HLGR in enterococci is previously known to be caused by one gene (*aac(6')-le-aph(2")-la*) encoding bifunctional enzyme *AAC(6')-APH(2")* in clinically useful aminoglycosides (Ferretti *et al*,1986). In recent years, three new aminoglycoside resistance genes (*aph(2")-1b*, *aph(2")-1c* and *aph(2")-1d*) that also mediate resistance to gentamicin have been detected in enterococci (Chow *et al*, 1997; Kao *et al*, 2000; Tsai *et al*, 1998). The *aac(6')-le-aph(2")-la* appears to have been conveyed to both enterococci and staphylococci via plasmid and transposon (Simjee and Gill,1997). Incidence of recent enterococci isolates showing HLGR varies depending on countries as well as medical facilities. In a large scale survey involving 27 European countries, the percentage of HLGR enterococci varied by country ranging from 1 to 49% and by species 19.7% *E.faecalis* and 13.6% *E.faecium* (Schouten *et al*, 1999). National survey in USA indicated the incidence of HLGR as 26% *E.faecalis* and 30% *E.faecium*. Another survey from European university hospitals reported the incidence of the strains

with HLGR being 32% *E.faecalis* and 22% *E.faecium* (Schmitz *et al,* 1999). In this study, the distribution of genes encoding four AMEs (ie *aac(6')-le-aph(2")-la*, *aph(2")-1b*, *aph(2")-lc* and *aph(2")-ld*) was investigated by multiplex PCR for 32 *E.faecalis* and 20 *E.faecium* isolates from RIE. 31% *E.faecalis* and 10% *E.faecium* were found to be resistant due to presence of *aac(6')-le-aph(2")-la* gene. However, the remaining three genes were not detected. The use of multiplex PCR for the dectection of AMEs was in line with the previous findings by Vakulenko *et al*, 2003. However, based on none detection of the remaining three genes, It could be suggested that there could be existence of the resistance mechanism other than AME including ribosomal mutation which have not been detected with the present techniques. Seven isolates (all *E.faecalis*) which were found to harbour AME gene were further screened for presence of Tn*5281* elements responsible for carrying and disseminating AME resistant gene and presence of IS*256* elements which flank the enzyme both sides in inverted repeat in line with previous findings by Schmitz *et al*, 1999; Simjee *et al*, 2000; Lyon,1984. The results established the presence of Tn5281 elements as well as IS*256* (Figs 6.2 and 6.3).

7.6 Alteration in *Gyr*A and *Par*C subunits in Quinolone Resistant enterococci

Resistance to fluoroquinolones appears to be due to mutations mediated via altered amino acids in QRDR of DNA gyrase subunit or topoisomerase 1V. Specific amino acids within *gyr*A or *par*C QRDR appear to correlate with sensitivity against quinolones because two sequences code for a specific part of DNA gyrase and topoisomerase 1V which may be, probably, the molecular targets of quinolone antibiotics. Previous studies by Korten *et al*, 1994; El Amin *et al*, 1999; Kanematsu *et al,* 1998) showed that mutation in *par*C of *E.faecalis* occurred at position 80 and 84; whereas the findings by Ferrero *et al*, 1994 showed that mutation in *par*C of *E.faecalis* occurred at position 83 and 87.

However, findings from this study showed that mutation in *par*C of *E.faecalis* occurred at position 80 and 87 for isolates with MIC>256mg/l and at position 80 and 85 for an isolate with MIC 0.5mg/l. These results appear to show variability with regards to positions of mutations in *par*C of *E.faecalis*. Owing to financial limitation, I was unable to sequence and confirm mutations in DNA gyrase among the selected isolates identified in PCR technique which could have detected *gyr*A along side *par*C mutations. However, the findings by Kanematsu *et al*, 1998 indicate that *gyr*A mutations in *E.faecalis* occur at 83 and 87 positions. Mutations are associated with a profound increase in the resistance to all quinolones. The factors which influence mutations of *par*C at positions 80 and 84 or 85 or 87 which indicate resistance may be due to topoisomerase 1V being the primary target (Kanematsu *et al*, 1998) based on the position of the mutation and the number of mutations involving types of amino acids. Mutations at *par*C positions 80 and 84 or 85 or 87 are usually more infuencial when the topoisomerase 1V is the main target, for example in *E.faecalis* (Kanematsu *et al*, 1998). *Par*C 80 codes for serine which appears to be direct quinolone binding site of the enzyme (Maxiwell, 1992). Mutation at this position is, therefore, appears to be the prerequisite for high resistance. Position 84 or 85 or 87 might be another binding site for quinolone as mutations at this position alone seems to have effect on quinolone sensitivity. Fluoroquinolone resistance appears to be higher when mutations occur at positions 80 and 84 or 87 than only one mutation in either 80 or 84 or 87. It appears as if in a very high quinolone resistance (Cip MIC>256mg/l), at least two mutations at *par*C 80 and 84 or 87 are needed (Ferrero *et al*, 1994). The effects of parC QRDR are insignificant on sensitivity. Mutations in QRDR not involved at position 80 and 84 or 87 do not play an important role on the level of resistance. It is assumed that two mutations at 80 and 84 or 87 for *par*C may be the most efficient way a bacterium can develop high resistance to quinolone drugs. Therefore, the

more mutations, the higher level of resistance can occur in gram-positive or gram-negative bacteria. In this study, 36 *E.faecalis* and 14 *E.faecium* were found to be resistant to ciprofloxacin with MICs >256mg/l. However only six isolates (all *E.faecalis*) were chosen for further examination and one of them had MIC of 0.5mg/l (sensitive) and the remainder had MICs of >256mgl each. PCR technique was used in identification of *gyr*A and *par*C in the isolates which were involved in the QRDR in line with the findings of Korten *et al,*1994; Piddock,1999; Kanematsu *et al*,1998). This study established the existence of *gyr*A or *par*C or both in all isolates identified by the PCR method. The products of PCR for *par*C were further sequenced to establish the amino acids involved in the mutations in *par*C. The results revealed that four isolates had mutation with a change of one amino acid each at Ser-80-Ile and two silent mutations while another had mutation with two changes of amino acids at Ser-80-Ile and Glu-110-Asp and two silent mutations. The sensitive isolate (MIC=0.5mg/l) had mutation at Ala-80-Val and a silent mutation. However, sensitive isolate had subsitituted Ala for Ser at position 80 and Val for Ile at position 80 of *par*C. The subsitituted amino acid appears not have any important role in the level of resistance but the loss of serine at position 80 that is most significant than the subsitituted amino acid because position 80 is a basic requirement for the bacterium to become resistant. This subsititution of amino acid (Ser) in a sensitive strain such as RIE-18B/314 could provide the strain with intrinsic resistance against ciprofloxacin. Four isolates (RIE-18B/376, RIE-18B/379, RIE-18B/390 and RIE-18B/911) had each one amino acid mutation (Ser-80-Ile) while one isolate (RIE-18B/414) had two mutations (Ser-80-Ile and Glu-110-Asp) in line with the previous findings by Kanematsu *et al*, 1998. However, the effect on resistant level of subsitituting amino acid in certain position of quinolone drug requires futher investigation.

7.7 PFGE Analysis and Interpretation

Several techniques have been used in medical microbiology for acquiring information on the spread of pathogenic bacteria within the hospital environment and outside in the community (Antonishyn *et al*, 2000). The PFGE technique used in this study was to analyze the degree of clonality with the gentamicin-resistant isolates (*E.faecalis* and *E.faecium*) obtained from RIE. The interpretation was done based on the criteria described by Tenover *et al,*(1995). The criteria are only used in the hospital laboratories when examining relatively small sets of isolates (ie not more than 30) which are related to putative outbreak of the disease. The interpretation criteria was used to show the number of fragment differences that would be expected within the PFGE patterns showing a defined number of genetic occurrences either as point mutation resulting in the creation or loss of restriction sites and insertions or deletion of DNA. Thus, PFGE patterns that differ by two or three fragments are deemed to be closely related on the differences that occur through a single genetic event. According to Tenover *et al*, 1995, two genetic events would result into four to six fragment differences showing unrelatedness. Tenover *et al*, 1995, proposed that these criteria are reliable if PFGE resolves at least ten distinct fragments. According to Tenover *et al,* 1995, a point mutation that would lead to the criteria of an additional restriction site may result in a three fragment differences. This might be taken as closely related to the original pattern. However, transfer of large chromosomal elements associated with vancomycin resistance could alter the biochemical properties of the strain (McAshan *et al,*1999). The study by Morrison *et al*, 1999 shows that a single strain can differ by up to seven fragments based on the

temporal, association of the isolates representing a single strain. If the criteria defined by Tenover *et al*, 1995, were widely accepted, they would not be applied to all bacterial species because it is recognized that different bacterial species vary in degree of polymorphism that they exhibit (Struelens *et al*, 1996). Although criteria defined by Tenover *et al*, 1995, provide some interpretations of PFGE patterns by allowing easier comparison of the conclusions reached by different epidemiology studies, it is upto the individual to accept conclusions drawn from such interpretation. It was unfortunate in this study that information related to the gentamicin resistant *E.faecalis* and *E.faecium* strains from each ward were not obtained at the time of collection from RIE in order to establish colonial or heterogeneous infections. The visual comparison of patterns and interpretation were done in this study based on criteria described by Tenover *et al*, 1995 for gentamicin resistant clinical isolates obtained from RIE. However, Diversity database analysis was undertaken based on images digitized from the original Polaroid photographs that were used for the original visual PFGE gels. Isolates which were deemed to be identical by viaual comparison of PFGE patterns had wide ranging percentage similarities according to Diversity Database analysis from Bionumeric Software Version 3.0 computer. The computer software identified isolate RIE-17B/849 and RIE-18B/33 as nearly identical (80% similarity) but failed to detect identity between RIE-17B/235 and 18B/149 (40% similarity). It also failed to identify between RIE-18B/662 and RIE-18B/749 (50% similarity). RIE-18B/517 and RIE-18B/567 identity with 76% similarity and closely related isolates RIE-18B/815 and RIE-19B/471 showed 68% similarity. Isolates RIE-18B/536 and RIE-18B/632 had only 66% similarity. All the isolates identified as unrelated to any other isolates by visual comparison of PFGE patterns were confirmed by computer software as unrelated. However, the computer software calculates that the two isolates have 66% similarity, such a percentage similarity

shows that the strains are not related although it may be that the percentage may be significant given the variety observed in the percentage similarity of identical strains.

7.8 Conclusion

The results presented in this thesis have demonstrated the existence of synergistic as well as antagonistic activities of the combined antibiotics against enterococcal clinical isolates (*E.faecalis* and *E.faecium*) obtained from RIE. All the clinical isolates were gentamicin resistant with the majority being *E.faecalis*. Owing to lack of information from each ward at RIE where clinical isolates were obtained for this study, the gentamicin resistance outbreak within the wards was not established. However, analysis by PFGE appears to show the diverse nature of the isolates in the wards of RIE. This study revealed the presence of AME resistance gene (*aac(6')-le-aph(2")-la*) responsible for HLGR among the isolates as well as Tn*5281* harbouring the resistance gene (*aac(6')-le-aph(2")-la*) and flanked by IS*256* in an inverted repeat on both sides of the gene. The presence of AMEs in Tn*5281*-like transposons flanked by IS256 in some isolates in this study with high level of gentamicin resistnce were overcome by antimicrobial combinations resulting in synergism. These are very interesting results but it might suggest that these isolates might be at the border between high-level resistance and moderate-level resistance to gentamicin although they had AME. Most of the isolates in this study were found to be resistant to ciprofloxacin (MIC= >256mg/l). This study also revealed the existence of *gyr*A and *par*C responsible for higher resistance among the isolates. Further sequencing of PCR products of *par*C of six selected *E.faecalis* (5 isolates with MIC= >256mg/l & one with MIC= 0.5mg/l) revealed the presence of mutations in QRDR at positions 80 and 87 with one amino acid change and two silent mutations for four isolates (MIC= >256mg/l) and one isolate with MIC= >256 but with two amino acids mutations at

position 80 and Glu-110-Asp and two silent mutations. Silent mutations were either at position 85 or 87 or both. Sensitive isolate (MIC=0.5mg/l) had mutation at 80 and 85 and Ala substituted ser at position 80 and Val substituted Ile at 80 which appeared to have played no role in the resistance of this isolate but showed intrinsicness against ciprofloxacin. The combination of syerncid and vancomycin revealed the presence of antagonistic activity of the combined drugs in this study and indicating that one of antimicrobial agents might have cancelled out or interfered with the activity of the other agent. The presence of AMEs in Tn*5281*-like transposons flanked by IS256 in some isolates in this study with high level of gentamicin resistnce were overcome by antimicrobial combinations resulting in synergism. These are very interesting results but it might suggest that these isolates might be at the border between high-level resistance and moderate-level resistance to gentamicin although they had AME. This study also revealed that some isolates tested carried both AME and *gyr*A or *par*C resistance genes or all which resulted into high-level resistance to both gentamicin and ciprofloxacin in this study. This suggests that these isolates may carry more than two resistance genes. The investigation by PFGE did not establish source of the outbreak of gentamicin-resistant isolates in RIE since information about the wards in which these isolates were collected was not available at the time of obtaining the isolates. However, similarity analysis showed a diverse background of the isolates.

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Appendix A API Profile and Haemolysis on BA*(α &β-Haemolysis)

Isolate #	Species	API Profile	Category Scores %	α or β-haemoloysis
17B/235	*E.faecium*	7 3 5 7 5 1 1	98.69	α
17B/377	*E.durans*	7 3 5 3 4 1 0	94.9	α
17B/407	*E.faecalis*	7 1 0 3 3 1 1	98.4	α
17B/452	*E.faecalis*	7 1 6 3 3 1 1	98.5	α
17B/686	*E.faecalis*	7 1 5 3 7 1 1	95.9	β
17B/721	*E.faecium*	7 1 5 7 5 1 0	97.1	α
17B/761	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
17B/819	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
17B/849	*E.faecium*	7 3 5 7 7 1 0	99.7	α
18B/33	*E.faecium*	7 3 5 7 7 1 0	99.7	α
18B/89	*E.faecium*	7 1 4 3 3 1 1	99.3	α
18B/149	*E.faecium*	7 1 6 7 5 1 0	81.2	α
18B/234	*E.faecium*	7 3 5 7 6 1 0	99.2	α
18B/254	*E.faecium*	7 1 7 7 7 1 1	98.3	α
18B/294	*E.faecium*	7 3 5 7 7 1 1	99.9	α
18B/298	*E.faecium*	7 1 7 7 7 1 1	98.3	α
18B/309	*E.faecalis*	7 1 4 3 7 1 1	99.2	β
18B/314	*E.faecalis*	7 1 4 3 7 1 1	99.2	α
18B/329	*E.faecalis*	7 1 4 3 7 1 1	99.2	α
18B/337	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
18B/358	*E.faecalis*	7 1 4 3 3 1 1	99.3	β

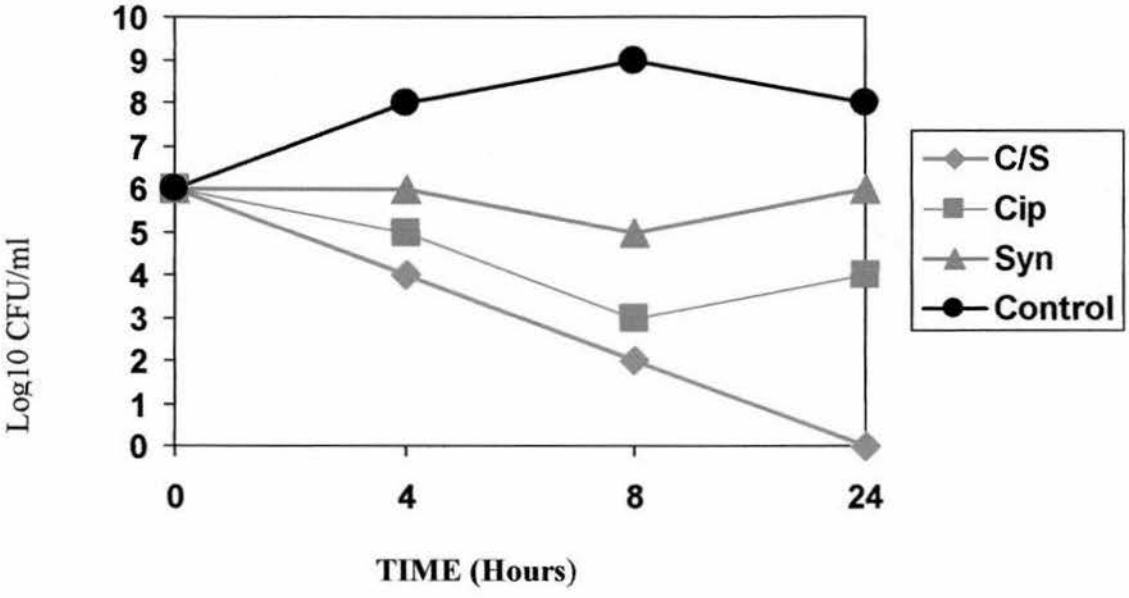
18B/376	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
18B/379	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
18B/382	*E.faecalis*	7 1 4 3 7 1 1	99.2	α
18B/387	*E.faecalis*	7 1 4 3 3 1 1	99.3	α
18B/390	*E.faecalis*	7 1 7 3 7 1 1	95.7	β
18B/414	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
18B/487	*E.faecium*	7 3 5 7 7 1 1	99.9	α
18B/506	*E.faecalis*	7 1 4 3 7 1 1	99.2	β
18B/517	*E.faecium*	7 3 5 7 7 1 0	99.7	α
18B/519	*E.faecalis*	7 1 4 3 7 1 1	99.2	α
18B/526	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
18B/536	*E.faecium*	7 3 5 7 5 1 0	98.6	α
18B/551	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
18B/555	*E.faecalis*	7 1 4 3 7 1 1	99.2	α
18B/556	*E.faecalis*	7 1 4 3 7 1 1	99.2	α
18B/567	*E.faecium*	7 1 7 7 7 1 1	98.3	α
18B/569	*E.faecalis*	7 1 4 3 7 1 1	99.2	β
18B/570	*E.faecalis*	7 1 4 3 7 1 1	99.2	β
18B/578	*E.faecalis*	7 1 4 3 7 1 1	99.3	β
18B/612	*E.faecalis*	7 1 5 3 7 1 1	95.9	α
18B/632	*E.faecium*	7 3 5 7 5 1 0	98.6	α
18B/642	*E.faecalis*	7 1 6 3 7 1 1	94.8	β
18B/643	*E.faecalis*	7 1 4 3 7 1 1	99.2	β
18B/647	*E.facalis*	7 1 4 3 7 1 1	99.2	β

18B/662	*E.faecium*	7 3 7 7 1 0	99.7	α
18B/713	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
18B/745	*E.faecalis*	7 1 4 3 7 1 1	99.2	α
18B/749	*E.faecium*	7 3 5 7 5 1 0	98.6	α
18B/777	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
18B/791	*E.faecalis*	7 1 7 3 7 1 1	95.9	α
18B/807	*E.faecalis*	7 1 7 3 7 1 1	95.9	β
18B/813	*E.faecium*	7 3 5 7 7 1 0	99.7	α
18B/815	*E.faecium*	7 3 5 7 7 1 0	99.7	α
18B/825	*E.faecalis*	7 1 4 3 7 1 1	99.2	β
18B/854	*E.faecalis*	7 1 7 3 3 1 1	93.6	β
18B/869	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
18B/900	*E.faecalis*	7 1 5 3 7 1 1	95.9	α
18B/911	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
18B/946	*E.faecalis*	7 1 7 3 3 1 1	88.9	β
18B/960	*E.faecium*	7 1 7 7 3 1 1	98.8	α
18B/976	*E.faecalis*	7 1 7 3 3 1 1	93.6	β
19B/082	*E.faecalis*	7 1 5 3 7 1 1	95.9	α
19B/183	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
19B/300	*E.faecalis*	7 1 7 3 7 1 1	95.9	β
19B/315	*E.faecalis*	7 1 7 3 7 1 1	95.9	α
19B/336	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
19B/343	*E.faecalis*	7 1 4 3 7 1 1	99.2	α
19B/369	*E.faecalis*	7 1 4 3 7 1 1	99.2	β

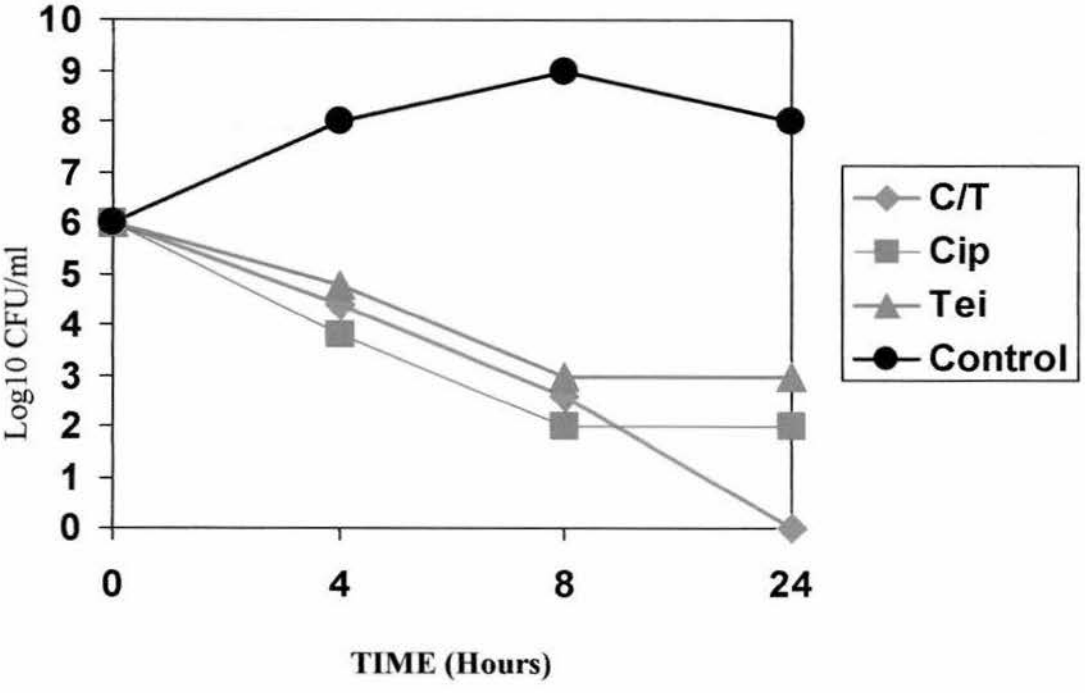
19B/391	*E.faecium*	7 3 5 7 7 1 1	99.9	α
19B/412	*E.faecalis*	7 1 4 3 7 1 1	99.2	α
19B/ 471	*E.faecium*	7 3 4 7 7 1 1	99.9	α
19B/491	*E.faecalis*	7 1 4 3 7 1 1	99.2	β
6155/6/95	*E.faecalis*	7 1 4 3 7 1 1	99.2	α
154/11/95	*E.faecalis*	7 1 4 3 3 1 1	99.3	β
ATCC 51299	*E.faecalis*	7 1 5 3 7 1 1	98.3	β
NCTC 12697	*E.faecalis*	7 1 4 3 7 1 1	99.2	α
NCTC 7171	*E.faecium*	7 1 5 7 7 1 0	98.8	α
NCTC 12202	*E.faecium*	7 1 5 7 5 1 1	94.9	α
D002	*E.faecium*	7 3 5 7 5 1 0	98.6	α
G051	*E.faecium*	7 3 7 7 7 1 1	99.9	α
G142	*E.faecium*	7 3 5 7 5 1 0	98.6	α
788/5/95	*E.faecium*	7 3 7 7 5 1 0	99.8	α

BA* = Blood Agar

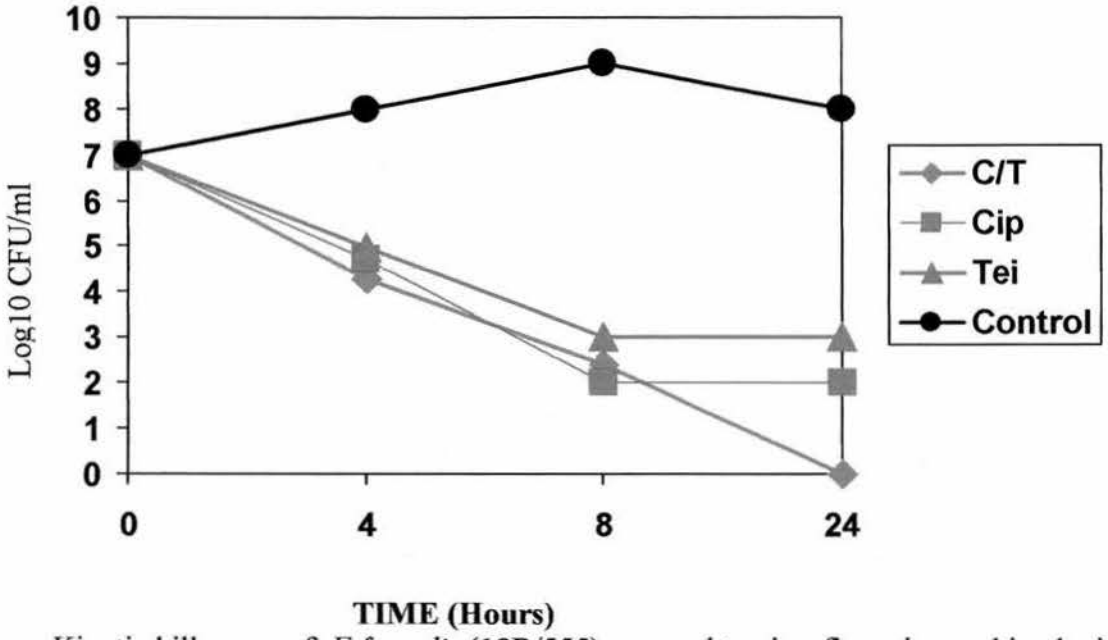
Appendix B. Kinetic-kill curves for *E.faecalis* and *E.faecaium*



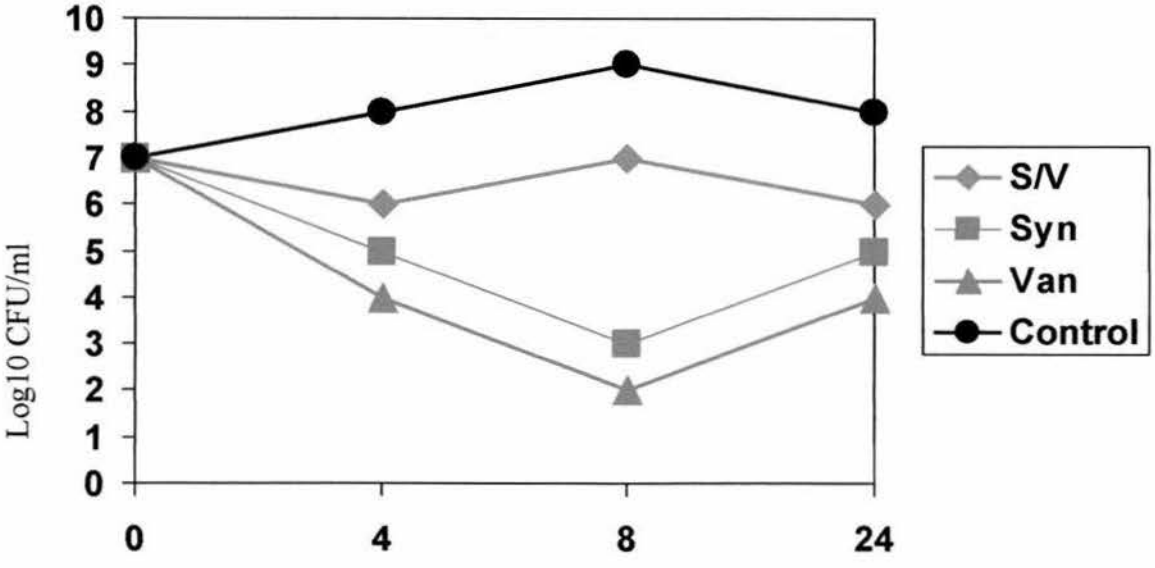
Kinetic kill curve of *E.faecium* (18B/298) exposed to ciprofloxacin combined with synercid



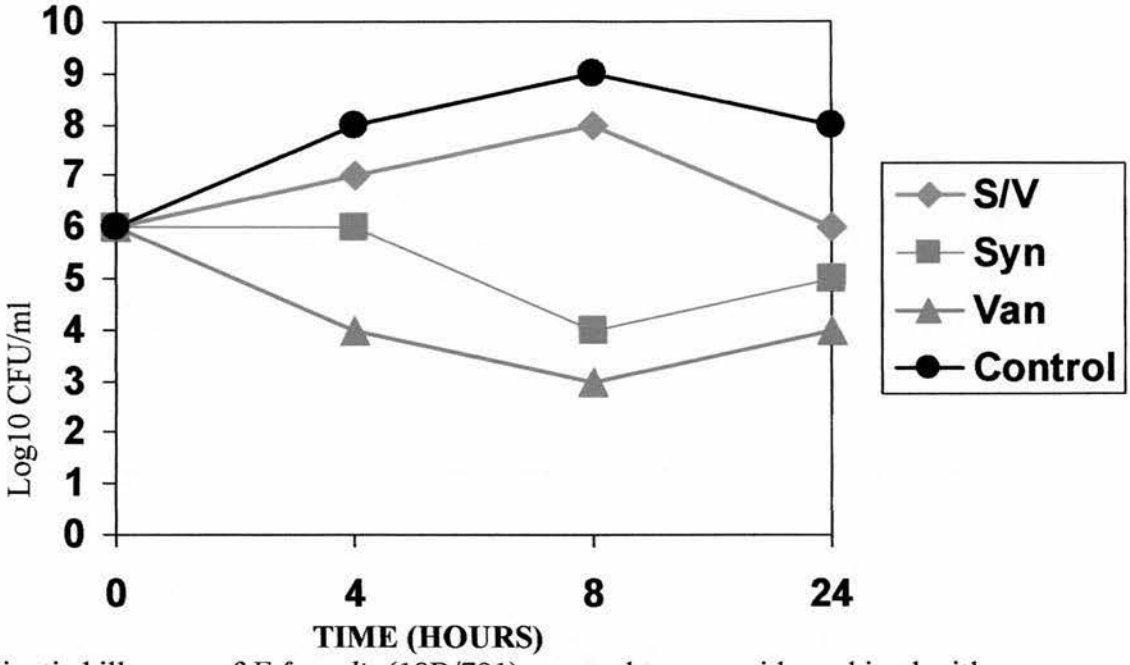
Kinetic kill curve of *E.faecium* (18B/749) exposed to ciprofloxacin combined with teicoplanin



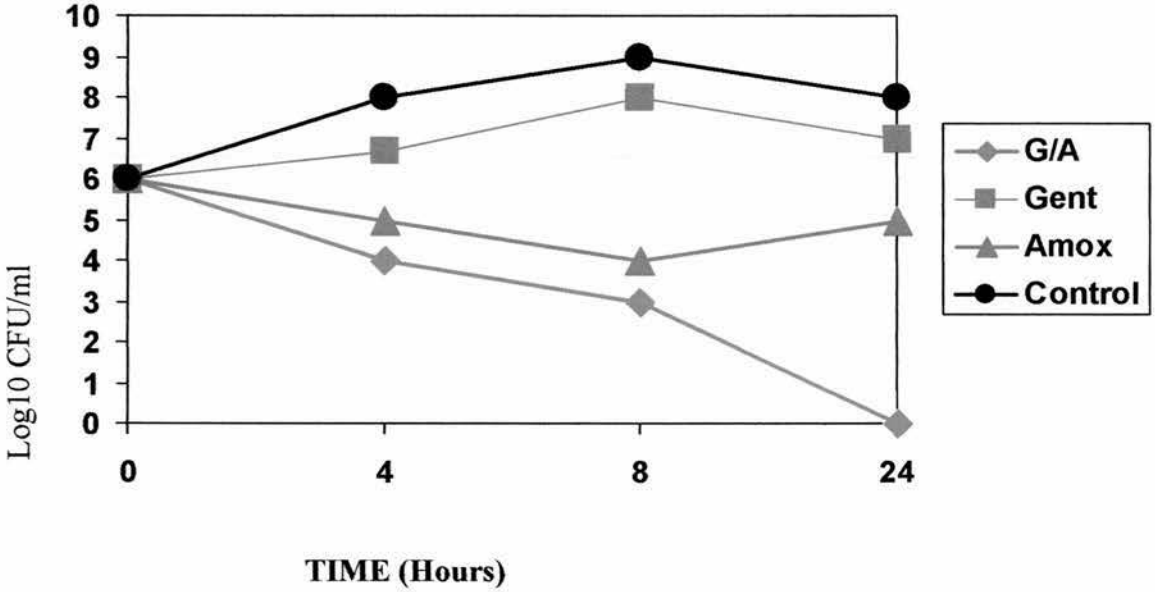
Kinetic kill curve of *E.faecalis* (18B/555) exposed to ciprofloxacin combined with teicoplanin



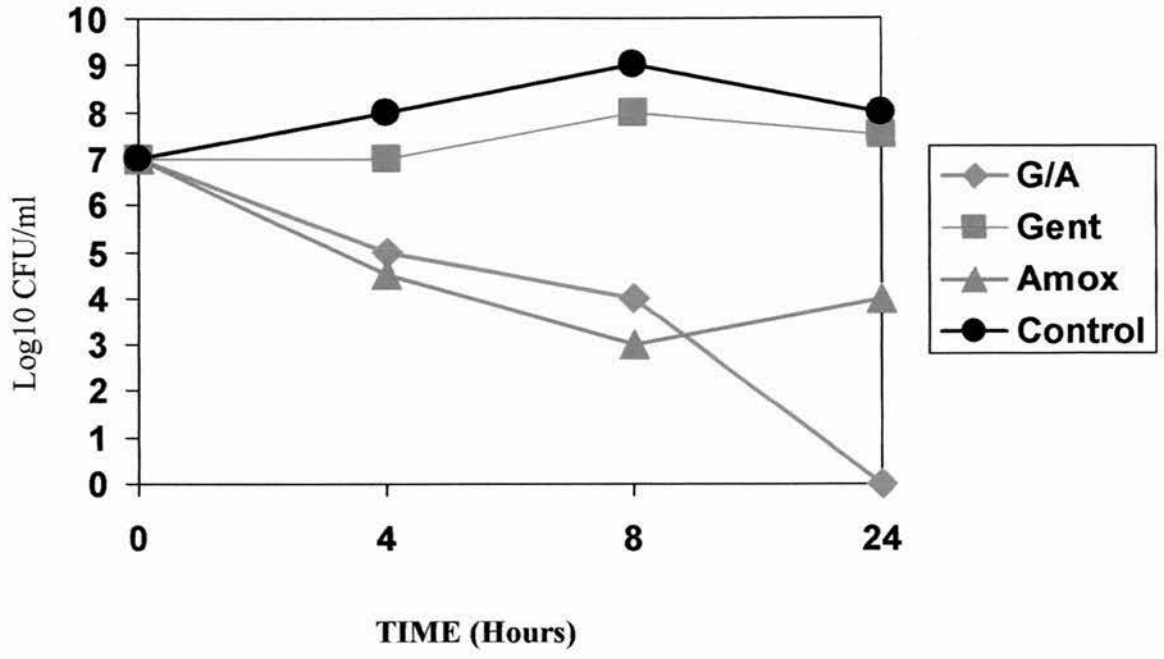
Kinetic kill curve of *E.faecium* (18B/632) exposed to synercid combined with vancomycin



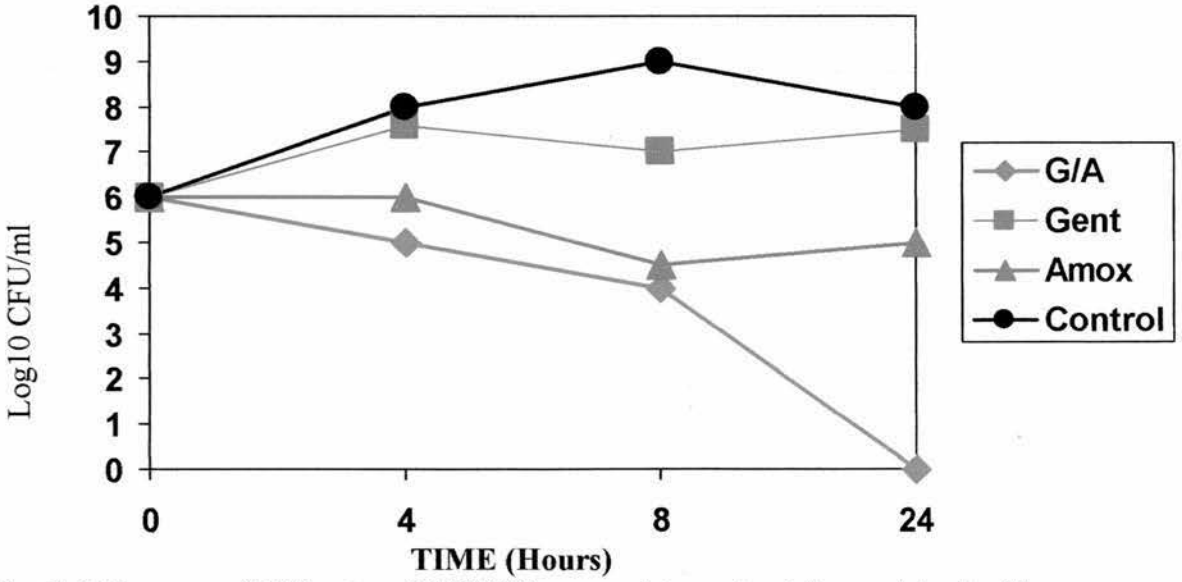
Kinetic kill curve of *E.faecalis* (18B/791) exposed to synercid combined with vancomycin



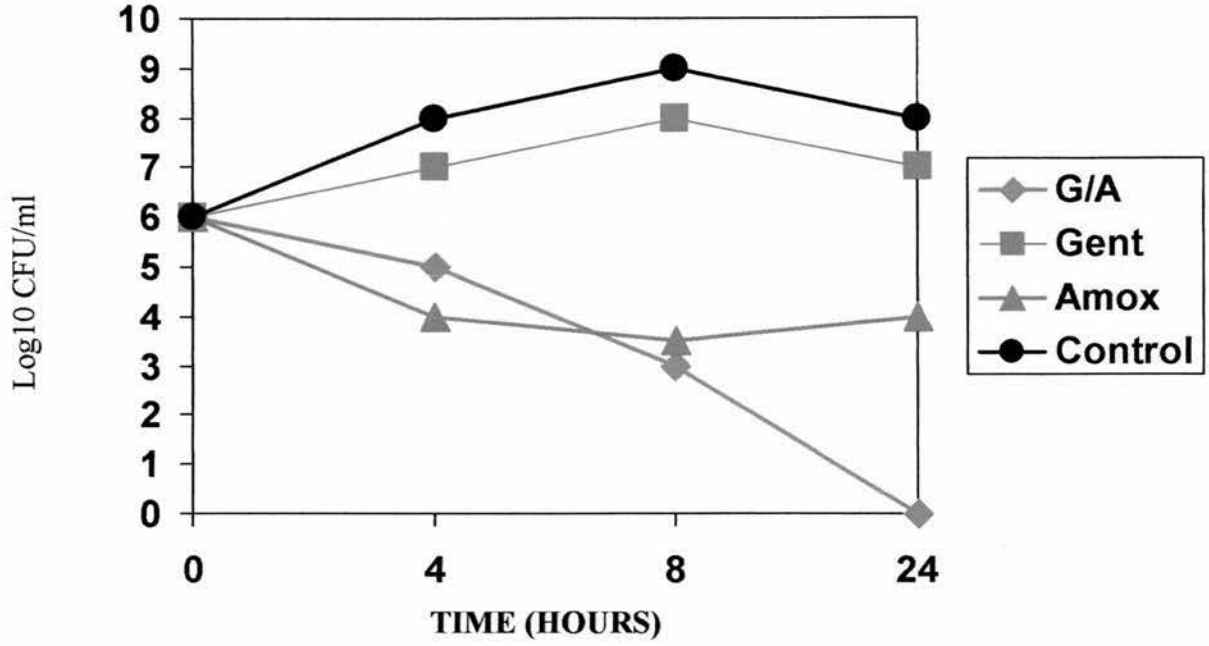
Kinetic kill curve of *E.faecalis* (17B/761) exposed to gentamicin combined with amoxicillin



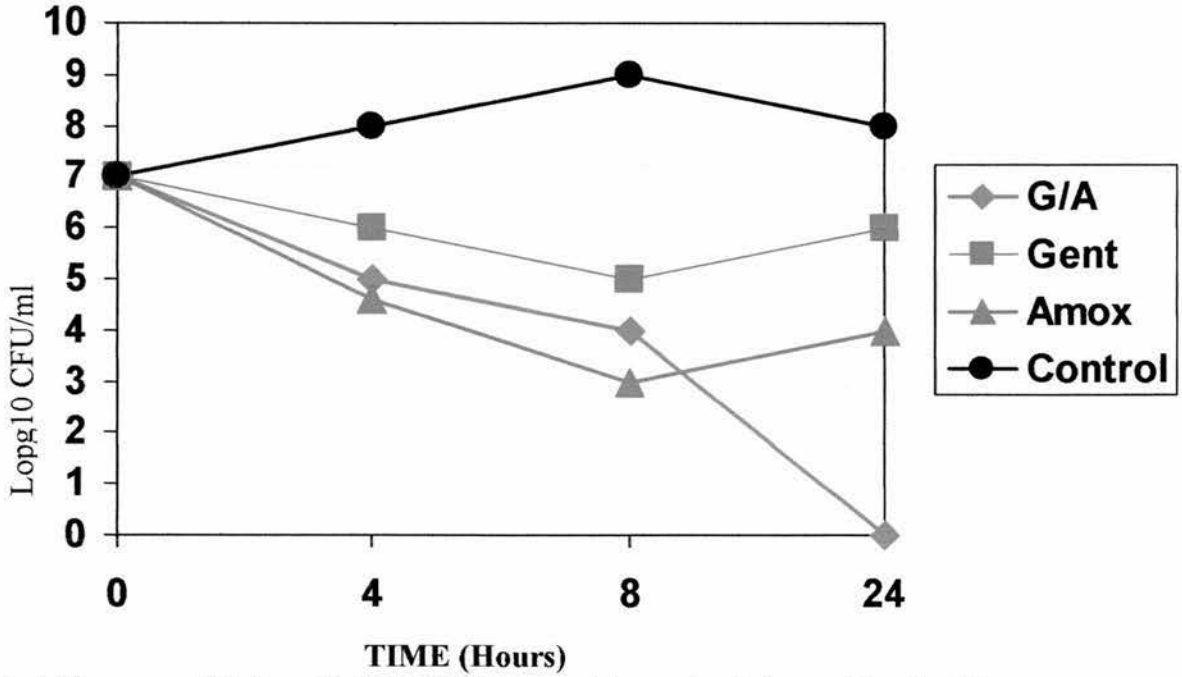
Kinetic kill curves of *E.faecalis* (17B/819) exposed to gentamicin combined with amoxicillin



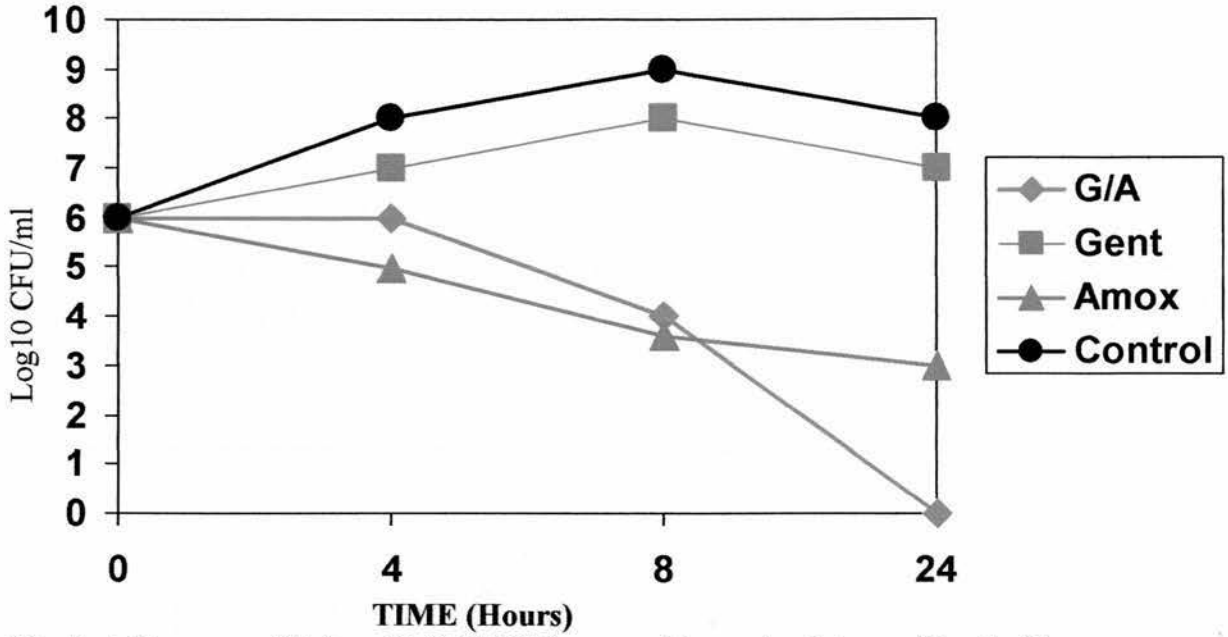
Kinetic kill curves of *E.faecium* (17B/849) exposed to gentamicin combined with amoxicillin



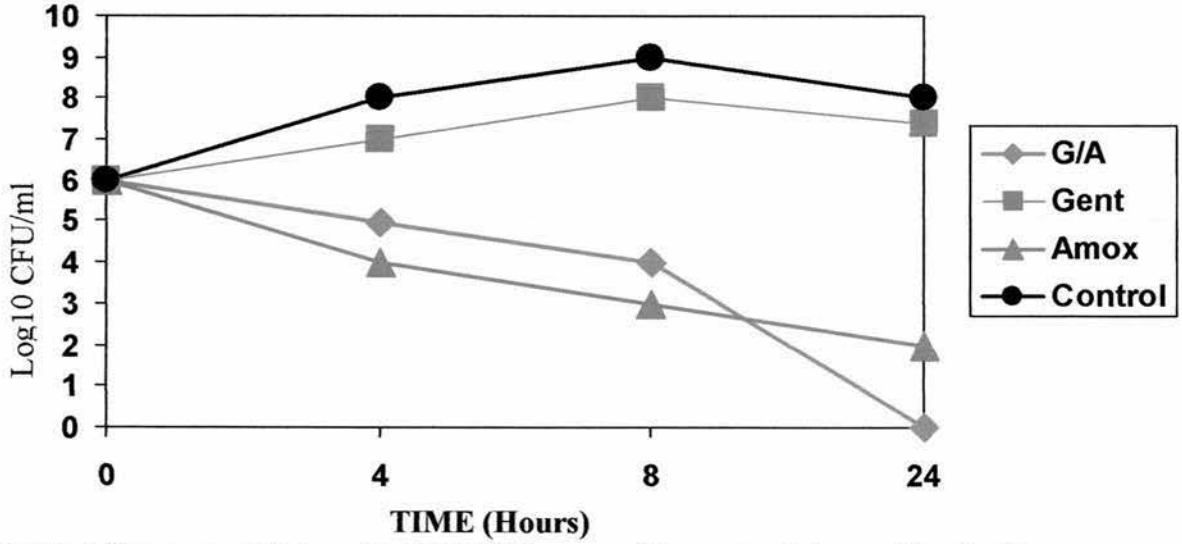
Kinetic kill curves of *E.faecalis* (18B/89) exposed to gentamicin combined with amoxicillin



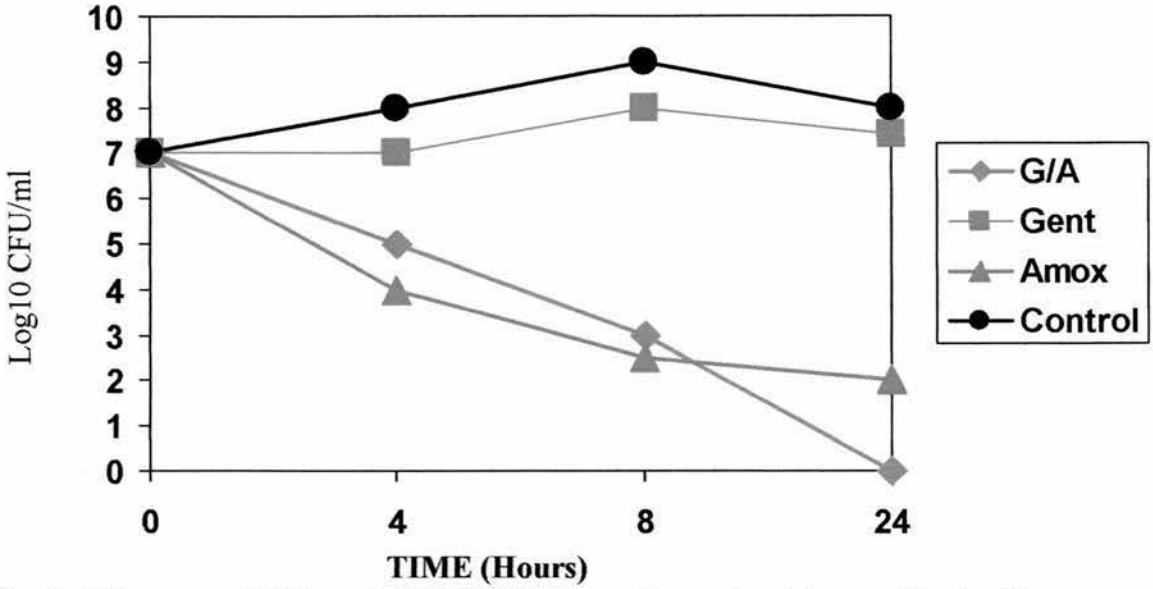
Kinetic kill curves of *E.faecalis* (18B/329) exposed to gentamicin combined with amoxicillin



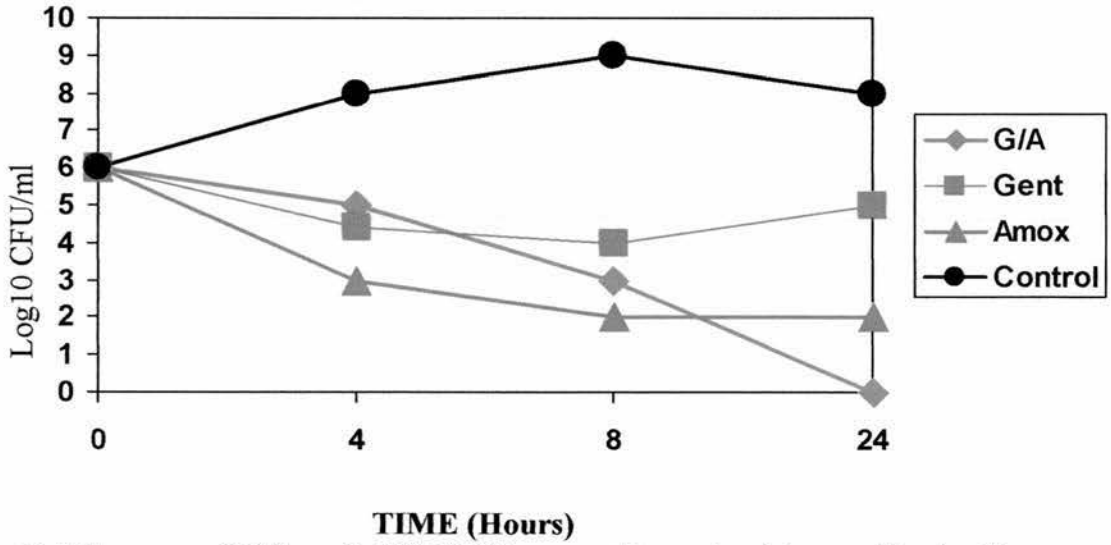
Kinetic kill curves of *E.faecalis* (18B/337) exposed to gentamicin combined with amoxicin



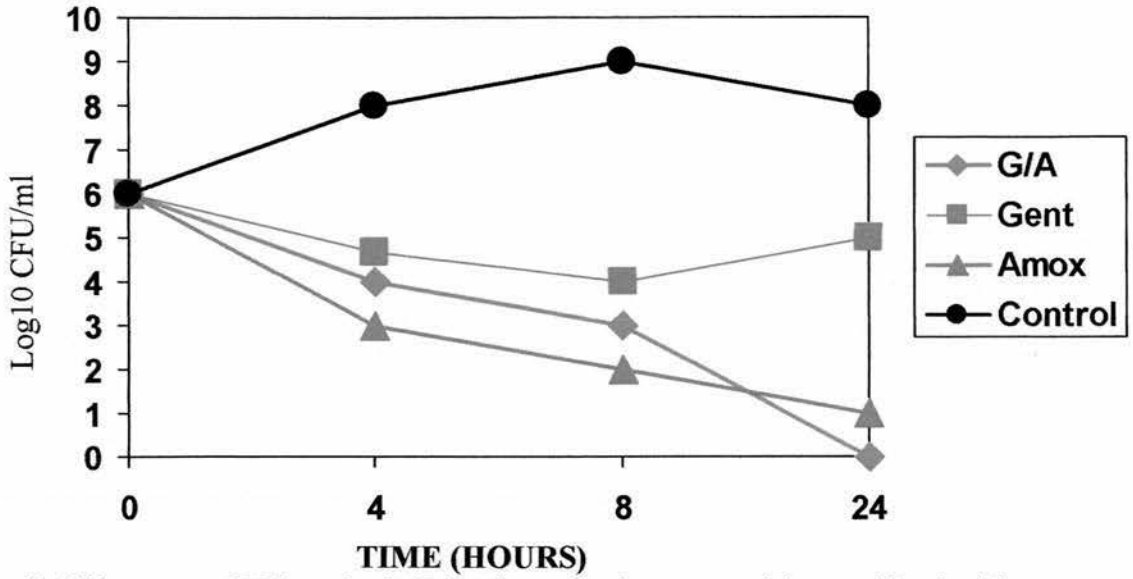
Kinetic kill curves of *E.faecalis* (18B/358) exposed to gentamicin combined with amoxicillin



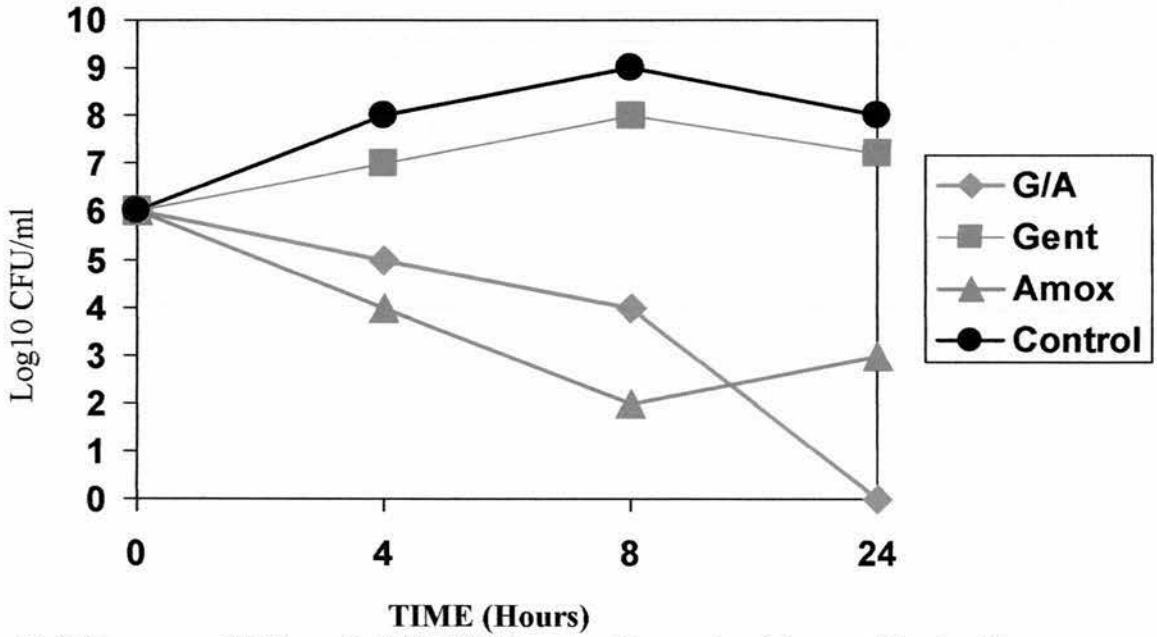
Kinetic kill curves of *E.faecalis* (18B/379) exposed to gentamicin combined with amoxicillin



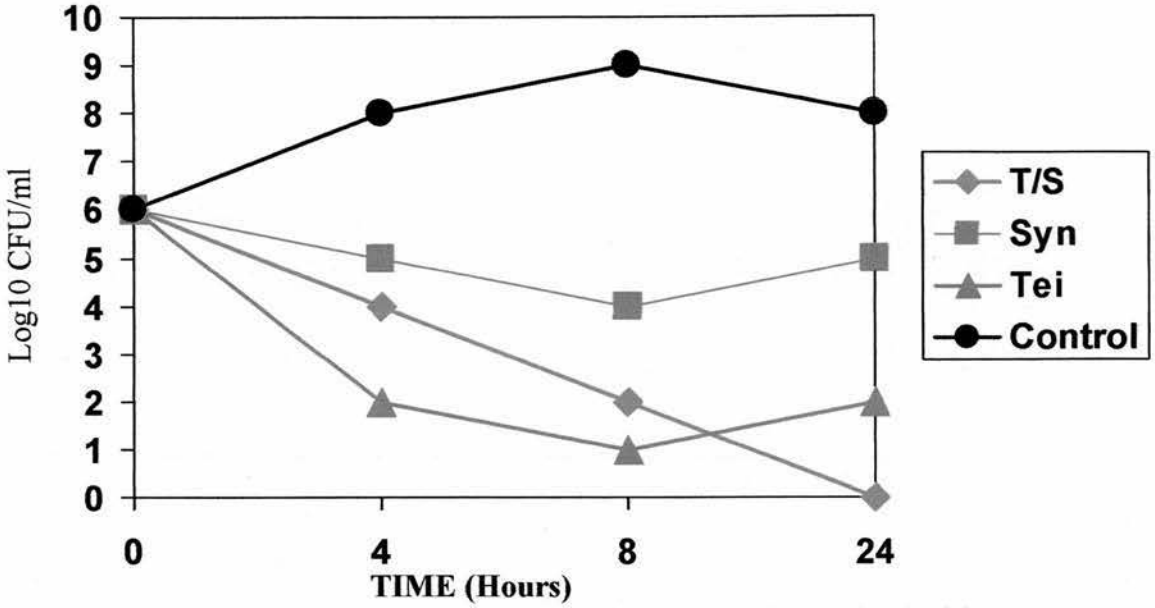
Kinetic kill curves of *E.faecalis* (18B/382) exposed to gentamicin combined with amoxicillin



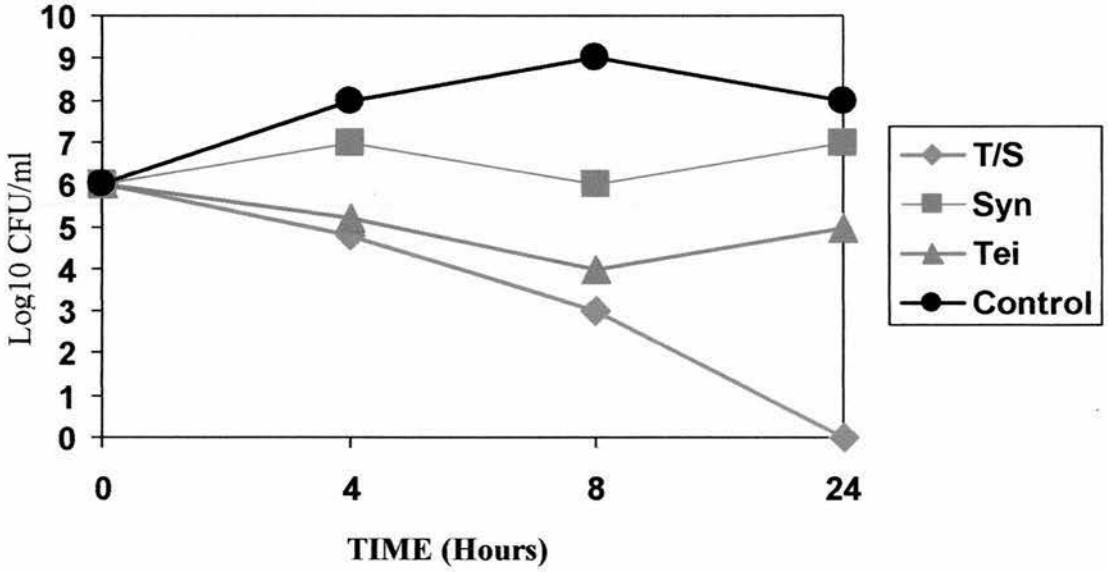
Kinetic kill curves of *E.faecalis* (18B/387) exposed to gentamicin combined with amoxicillin



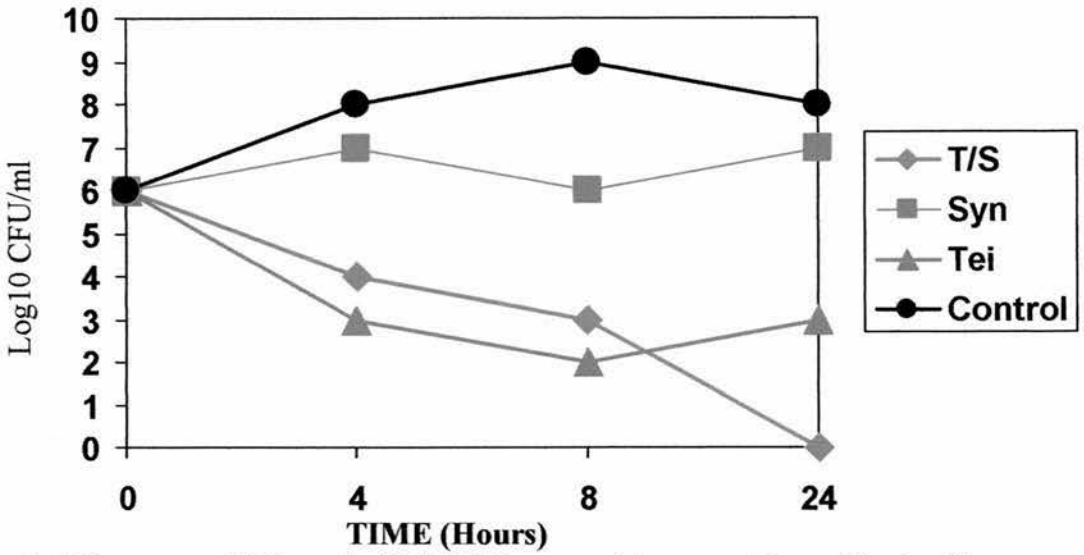
Kinetic kill curves of *E.faecalis* (18B/390) exposed to gentamicin combined with amoxicillin



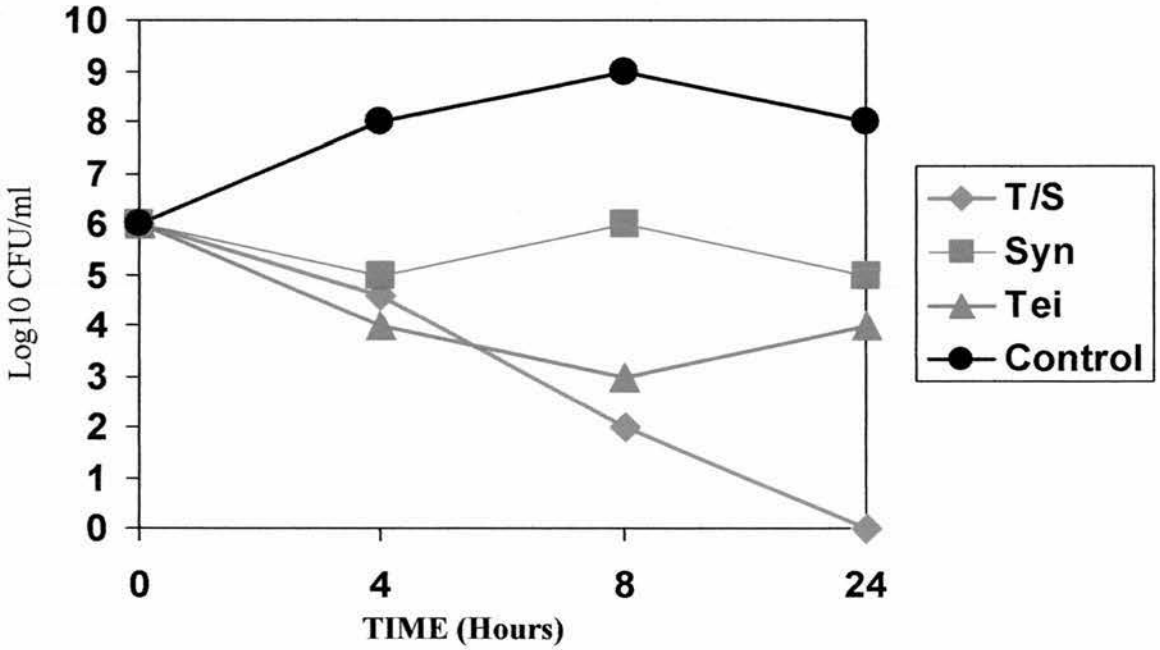
Kinetic kill curves of *E.faecium* (17B/235) exposed to synercid combined with teicoplanin



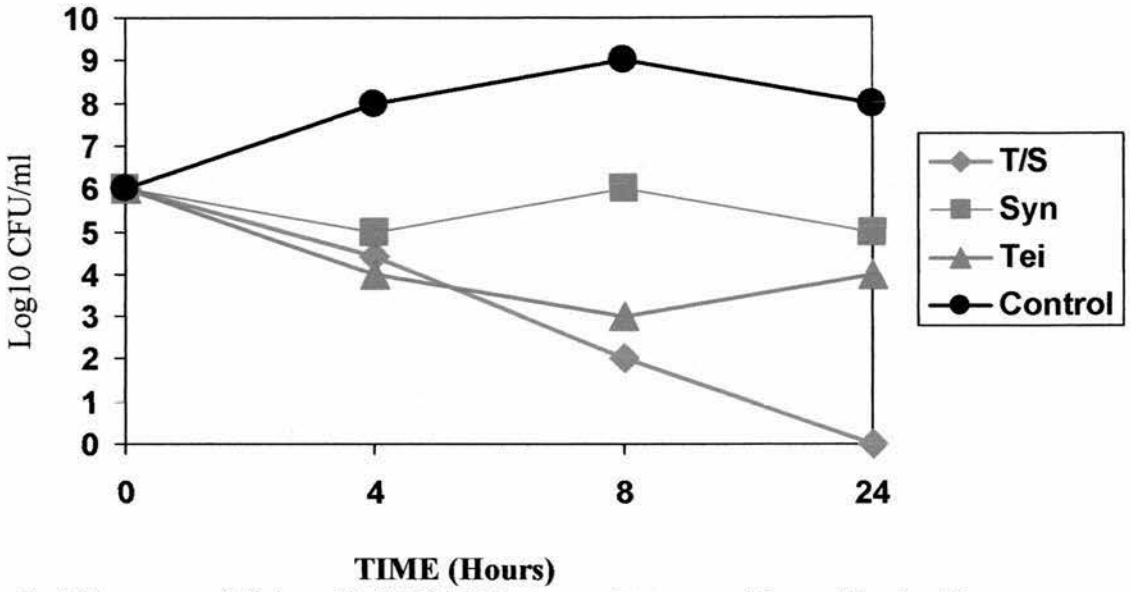
Kinetic kill curves of *E.faecalis* (17B/452) exposed to synercid combined with teicoplanin



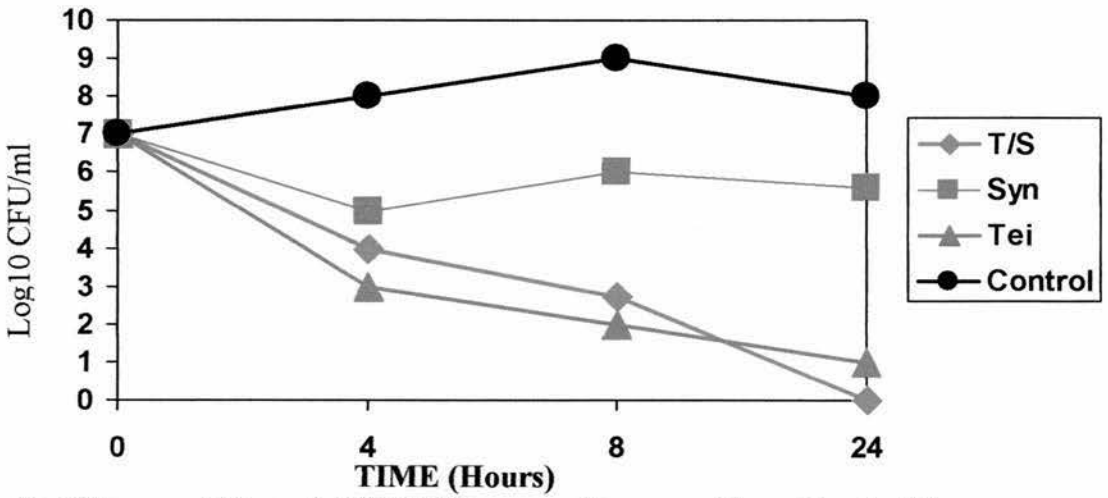
Kinetic kill curves of *E.faecalis* (17B/407) exposed to synercid combined with teicoplanin



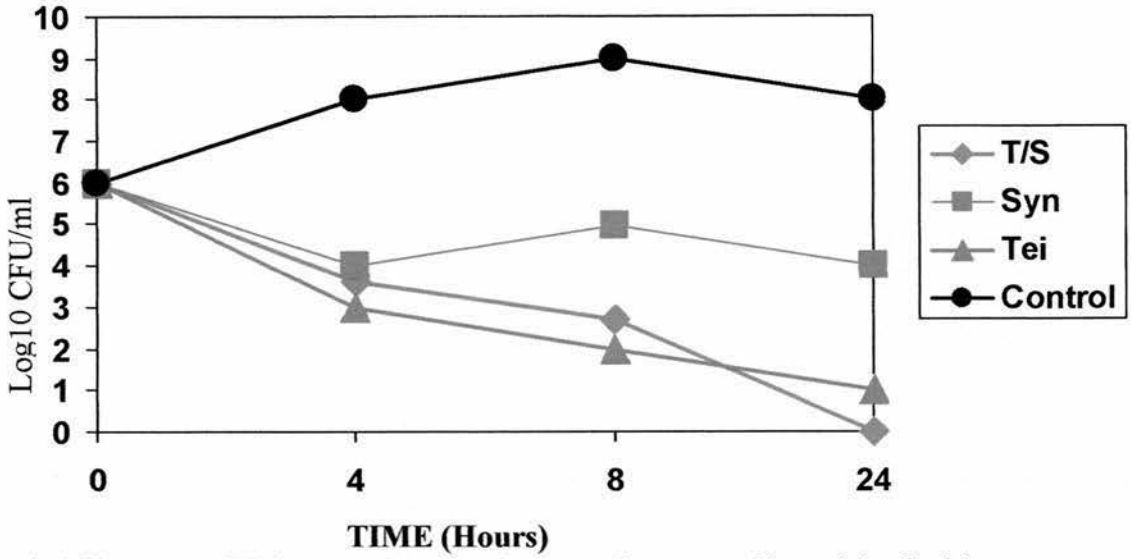
Kinetic kill curves of *E.faecium* (17B/721) exposed to synercid combined with teicoplanin



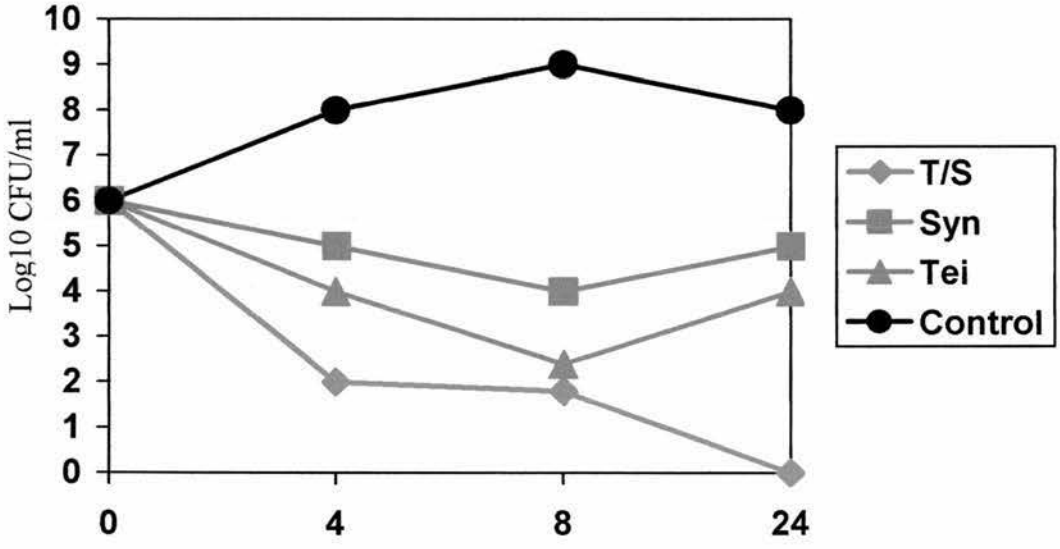
Kinetic kill curves of *E.faecalis* (17B/761) exposed to synercid combined with teicoplanin



Kinetic kill curves *E.faecalis* (17B/819) exposed to synercid combined with teicoplanin



Kinetic kill curves of *E.faecium* (17B/849) exposed to synercid combined with teicoplanin

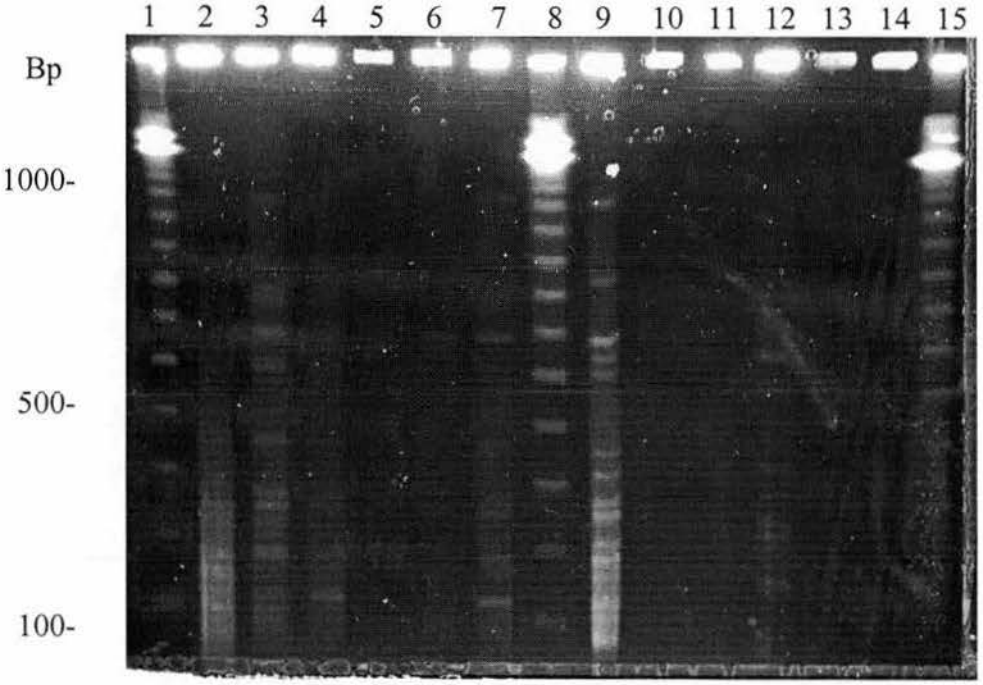


TIME (Hours)

Kinetic kill curves of *E.faecium* (D002) exposed to synercid combined with teicoplanin

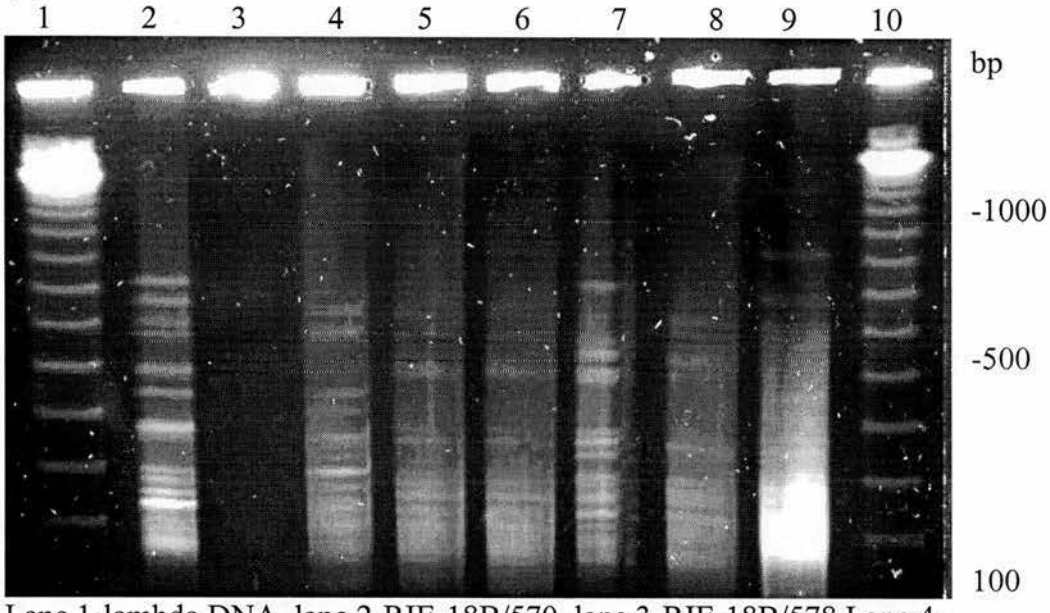
Appendix C. (a), (b), (c) & (d) PFGE patterns depicted for *E.faecalis* and *E.faecium*

(a)



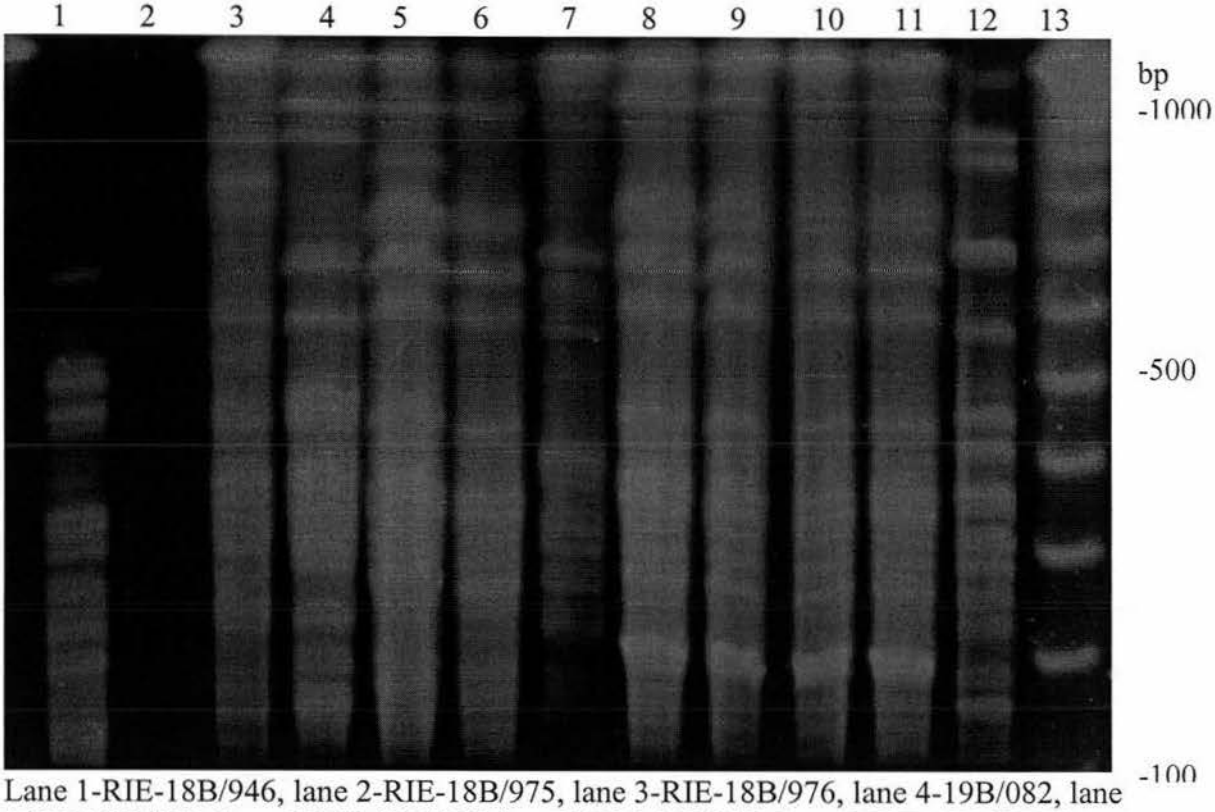
Lane 1-lambda DNA, lane 2-RIE-18B/382, lane 3-RIE-18B/387, lane 4-RIE-18B/390, lane 5-RIE-18B/414, lane 6-RIE-18B/487, lane 7-RIE-18B/506, lane 8-lambda DNA, lane 9-RIE-18B/519, lane 10-RIE-18B/526, lane 11-RIE-18B/551,lane 12-RIE-18B/555, lane 13-RIE-18B/556, lane 14-RIE-18B/569, lane 15-lambda DNA

(b)



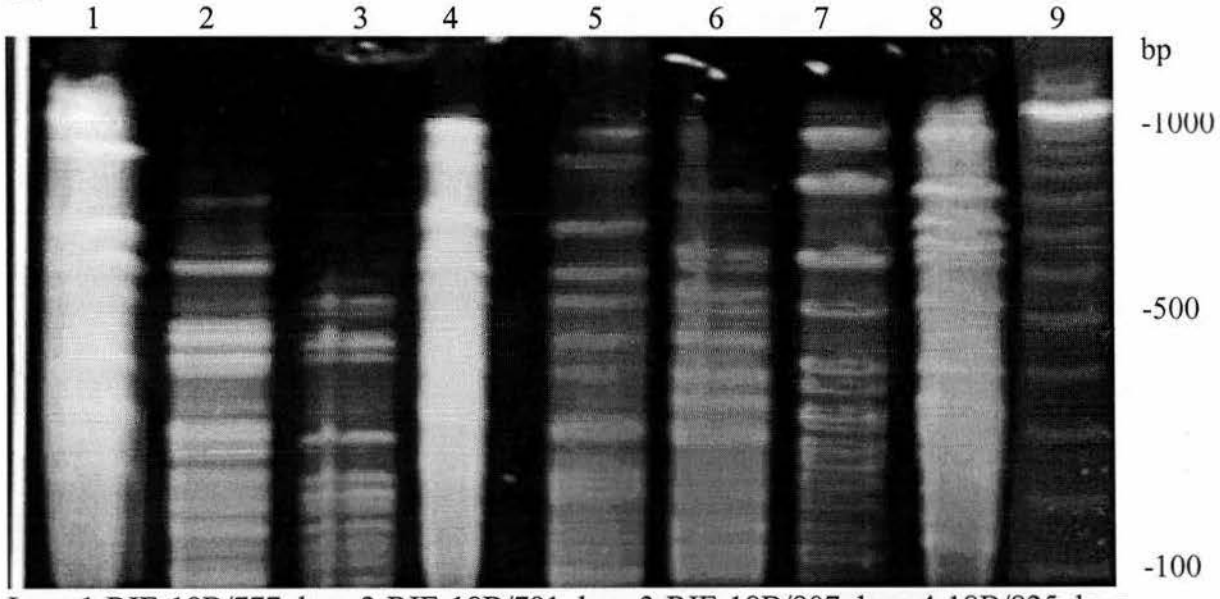
Lane 1-lambda DNA, lane 2-RIE-18B/570, lane 3-RIE-18B/578,Lane 4-RIE-18B/612, lane 5-RIE-18B/642, lane 6-RIE-18B/643, Lane 7-RIE-18B/647, lane 8-RIE-18B/713, lane 9-RIE-18B/745, lane 10-lambda DNA

(c)



Lane 1-RIE-18B/946, lane 2-RIE-18B/975, lane 3-RIE-18B/976, lane 4-19B/082, lane 5-RIE-19B/183, lane 6-RIE-19B/300, lane 7-RIE-19B/315, lane 8-RIE-19B/336, lane 9-RIE-19B/343, lane 10-RIE-19B/369, lane 11-RIE-19B/412, lane 12-RIE-19B/491, lane 13-λ lambda DNA

(d)



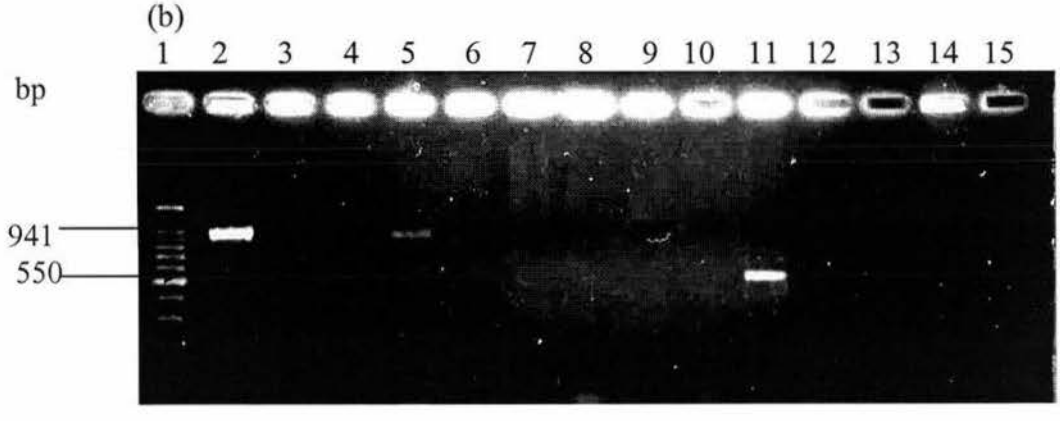
Lane 1-RIE-18B/777, lane 2-RIE-18B/791, lane 3-RIE-18B/807, lane 4-18B/825, lane 5-RIE-18B/854, lane 6-RIE-18B/869, lane 7-RIE-18B/900, lane 8-18B/911, lane 9-λ lambda DNA

Appendix D. Analysis of agarose gel electrophoresis of amplified ddl *E.faecium* and ddl *E.faecalis* PCR Products

(a)

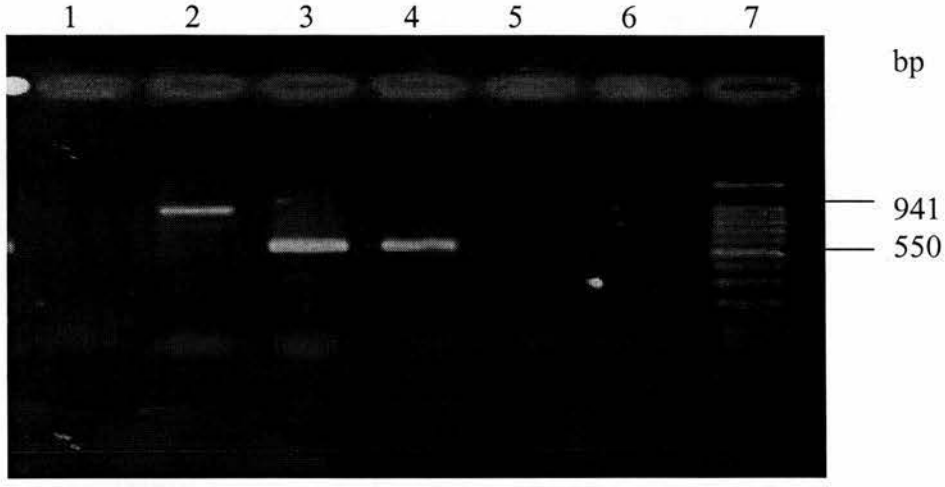
Lane 1-lambda DNA, lane 2-RIE-18B/379, lane 3-RIE-18B/382, lane 4-RIE-18B/387, lane 5-RIE-18B/390, lane 6-RIE-18B/414, lane 7-RIE-18B/487, lane 8-RIE-18B/506, lane 9-RIE-18B/517, lane 10-RIE-18B/519, lane 11-RIE-18B/18B/526, lane 12-RIE-18B/551, lane 13-RIE-18B/555, lane 14-RIE-18B/556, lane 15-RIE-18B/536, lane 16-lambda DNA

(b)



Lane 1-λ DNA, lane 2- RIE-18B/569 lane 3-RIE-18B/567, lane 4-RIE-18B/570, lane 5-RIE-18B/578, lane 6-RIE-18B/612, lane 7-RIE-18B /632, lane 8-RIE-18B/642, lane 9-RIE-18B/643, lane 10-RIE-18B/647, lane 11-RIE-18B/662, lane 12-RIE-18B/710, lane 13-RIE-18B/745, lane 14-RIE-18B/749, lane 15-RIE-18B/777

(c)



Lane 1-RIE-18B/791, lane 2-RIE-18B/807, lane 3-RIE-18B/813,
Lane 4-RIE-18B/815, lane 5-RIE-18B/825, lane 6-RIE-18B/854
Lane 7-λ DNA